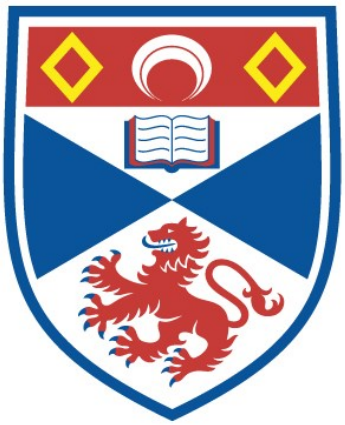
OSMOREGULATORY ROLE AND CONTROL OF SECRETION OF 1A-HYDROXYCORTICOSTERONE IN THE LESSER SPOTTED DOGFISH 'SCYLIORHINUS CANICULA'

Kenneth John Armour

A Thesis Submitted for the Degree of PhD
at the
University of St Andrews



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Osmoregulatory Role and Control of Secretion of 1α-hydroxycorticosterone in the Lesser Spotted Dogfish, *Scyliorhinus canicula*

by

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in the University of St. Andrews

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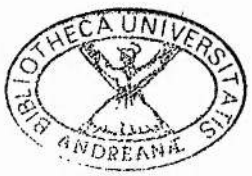

Declaration

a) I Kenneth John Armour, hereby certify that this thesis has been composed by myself, that it is a record of my own work, and that it has not been accepted in partial or complete fulfilment of any other degree or professional qualification.

Signed Date 20/12/90

b) I was admitted to the Faculty of Science of the University of St. Andrews under Ordinance General No. 12 in October 1987 and as a candidate for the degree of Ph.D. in October 1987.

Signed Date 20/12/90

c) I hereby certify that the candidate has fulfilled the conditions of the Resolution and Regulations appropriate to the degree of Ph.D.

Signature of Supervisor Date

21st December 1990.

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This thesis is dedicated to my mother,
Mrs. I. Armour.

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Finally, I would like to thank my parents for their support and encouragement during my studies.

ABSTRACT

1. Groups of dogfish, Scyliorhinus canicula, were adapted to either a high or a low protein diet over a period of 30 days. The fish readily ate both diets and maintained body weights.

2. Dietary adapted fish were acclimated to 130%, 100% and 50% seawater and the metabolic clearance rates (MCR) and blood production rates (BPR) for urea and 1α-hydroxycorticosterone (1α-OH-B) determined.

3. In 100% seawater low protein diet (LPD) fish had significantly lower urea BPR and MCR than the high protein diet (HPD) fish.

4. In 50% seawater plasma osmolality, sodium, chloride and urea concentrations decreased in both dietary groups. Plasma 1α-OH-B concentration and BPR were significantly increased and in HPD fish this was accompanied by an increase in 1α-OH-B MCR. The increase in plasma 1α-OH-B concentration was significantly greater in the HPD fish than in the LPD fish.

5. In 130% seawater plasma osmolality was increased in both dietary groups. In HPD fish this was achieved by increasing plasma urea, sodium and chloride concentrations. In LPD fish this was achieved by significantly elevating plasma sodium concentration only. Plasma 1α-OH-B concentration and BPR were significantly increased and MCR significantly decreased in LPD fish, but remained unaltered in HPD fish.

6. An isolated perifused interrenal gland preparation was developed for Scyliorhinus canicula, which produced a constant 1α-OH-B basal secretion rate after two hours and remained viable for more than 22 hours.

7. ACTH, AII, ANP, AVT, forskolin, dibutyryl cAMP and dibutyryl cGMP stimulated interrenal steroidogenesis.

8. ACTH-induced steroidogenesis was unaffected by the presence of dantrolene and verapamil but significantly increased in the presence of cholera toxin and significantly decreased in the absence of extracellular calcium.

9. Ile^5-AII-induced steroidogenesis was unaffected by the presence of verapamil but significantly decreased in the presence of dantrolene and in the absence of extracellular calcium.

10. Increases in potassium concentration were significantly steroidogenic only at 28mM and alterations in sodium concentration had no effect.

Abbreviations

1α-OH-B	1α-hydroxycorticosterone
AI	angiotensin I
AII	angiotensin II
AIII	angiotensin III
ACE	angiotensin converting enzyme
ACTH	adrenocorticotropin
ADP	adenosine bisphosphate
AHP	adenohypophysis
AMP	adenosine monophosphate
ANP	atrial natriuretic peptide
AS	argininosuccinate
ATP	adenosine trisphosphate
ATPase	adenosine trisphosphatase
AVT	arginine vasotocin
BPR	blood production rate
BSA	bovine serum albumin
Bt_2	dibutyryl
Ca^{2+}	calcium ion
cAMP	cyclic adenosine 3',5'-monophosphate
CCK	cholecystokinin
cGMP	cyclic guanosine 3',5'-monophosphate
CP	carbamoylphosphate
CPS	carbamoylphosphate synthetase
CTx	cholera toxin
CRF	corticotropin releasing factor
CRH	corticotropin releasing hormone
d.p.m.	disintegrations per minute

DG	1,2-diacylglycerol
DNA	deoxyribonucleic acid
ECF	extracellular fluid
EDTA	ethylenediamine tetra-acetic acid
EGTA	ethylenglycol-bis-(β-aminoethyl ether) tetra-acetic acid
ER	endoplasmic reticulum
FSH	follicle-stimulating hormone
G	guanine nucleotide-binding protein
GDP	guanosine bisphosphate
GFR	glomerular filtration rate
GH	growth hormone
GS	glutamine synthetase
GTH	gonadotropic hormone
GTP	guanosine trisphosphate
GTPase	guanosine trisphosphatase
Hepes	N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid]
HPD	high protein diet
ICF	intracellular fluid
IP_3	inositol 1,4,5-trisphosphate
LH	luteinising hormone
LPD	low protein diet
LPH	lipotropin
MCR	metabolic clearance rate
mRNA	messenger ribonucleic acid
MS222	tricaine methanosulphate
MSH	melanotropin

NAG	N-acetylglutamate
NHP	neurohypophysis
NPY	neuropeptide Y
OTC	ornithine transcarbamylase
β-PDH	β-pigment-dispersing hormone
PIP_2	phosphatidylinositol 4,5-bisphosphate
RIA	radioimmunoassay
S.D.	standard deviation
S.E.M.	standard error of the mean
TMAO	trimethylamine N-oxide
TRH	thyrotropin releasing hormone
TSH	thyrotropin
UI	urotensin I
UII	urotensin II
UFR	urine flow rate
VIP	vasoactive intestinal peptide

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1. GENERAL INTRODUCTION

1.1 Homeostasis

The ability to maintain a constant internal environment in the face of environmental variation has been the cornerstone of vertebrate evolutionary success. An integral component of this "homeostasis" (Cannon, 1929) is the maintenance of body fluid and electrolyte balance, known as osmoregulation.

Body fluids can be divided into intracellular and extracellular fluids, respectively, the chemical compositions of which are relatively uniform throughout the vertebrates. Intracellular fluid is rich in protein and potassium ions whereas extracellular fluid is rich in sodium and chloride ions and low in protein. With certain notable exceptions, vertebrates maintain their osmolalities at preset levels, approximately 25-30% of contemporary seawater, regardless of their environment.

The mechanisms of vertebrate body fluid homeostasis are similar throughout the group. The kidney is ubiquitous and, depending on the class, functions in conjunction with a variety of extra-renal regulators including the gills, gut, urinary bladder, gall bladder, skin, cloaca and salt glands to maintain body fluid volume and composition. The precise nature of osmoregulation within vertebrate groups depends, to a large extent, on their habitat.

1.1.1 Terrestrial Environment

The greatest physiological threat to life on land is dehydration. Water loss may result from evaporation from the body surface, respiratory surfaces, excretion in the urine and faeces and from sweating and panting.

Evaporative water loss can be minimised by decreasing skin permeability and by nasal exhalation. Urinary loss is minimised in mammals by the production of urine which is hyper-osmotic to the plasma and in birds and reptiles by the production of almost water-free urine.

1.1.2 Freshwater Environment

Vertebrates in freshwater, due to the osmotic difference between the dilute external medium and the more concentrated body fluids, face incipient water gain. Water accumulation across permeable membranes such as fish gills and amphibian skin is offset by the copious production of urine which is dilute with respect to the plasma. Sodium and chloride ions are actively reabsorbed across the gills of fish (Maetz, 1971) and by the skin of amphibians (Middler, Kleeman and Edwards, 1968; Bentley, 1973).

1.1.3 Seawater Environment

Marine vertebrates exhibit one of two osmoregulatory strategies: they either maintain their body fluids iso- or hyper-osmotic to the environment, or regulate hypo-osmotically with plasma osmolality 25-30% that of seawater. Hypo-osmotic regulators gain solutes and lose water across permeable membranes. Water loss in marine teleosts is offset by drinking large volumes of water (Smith, 1930) and excess salt is excreted across the gills and excess magnesium via the kidneys (Keys and Willmer, 1932). Cephalic salt glands secrete excess sodium and chloride in marine reptiles and birds (Schmidt-Nielsen, 1960) whereas marine mammals produce a hyper-osmotic urine.

Marine vertebrates maintaining iso- or hyper-osmotic plasma include some cyclostomes, elasmobranchs, holocephalans, coelacanths and a single amphibian. The cyclostomes consist of two distinct groups; lampreys inhabit both seawater and freshwater and apparently osmoregulate like euryhaline teleosts; hagfish are strictly marine and stenohaline and are considered the only true vertebrates to maintain body sodium and chloride concentrations similar (but not identical) to those of seawater. Osmotically, hagfish behave like marine invertebrates and are considered to be osmoconformers with limited but specific ionic regulation (Stolte and Schmidt-Nielsen, 1978). Urea retention occurs in all other classes that maintain extracellular fluid iso- or hyper-osmotic to the environment. Elasmobranchs, holocephalans and the coelacanth, Latimeria, maintain elevated plasma sodium concentrations relative to marine teleosts but body fluids are rendered hyper-osmotic to seawater by the retention of urea and trimethylamine oxide. Water loss is minimised by this hyper-osmolality but considerable ionic regulation occurs at the gills and kidney and also, in elasmobranchs, at the rectal gland (see section 2.2.4).

1.2 Nitrogen and Osmoregulation

In vertebrates a close relationship exists between osmoregulation and excess nitrogen elimination. The majority of nitrogenous waste arises from amino acid and protein catabolism although some waste is also formed from the breakdown of nucleic acids. The nitrogen produced by

the catabolism of amino acids and proteins is concentrated into one of three forms of nitrogenous waste, depending on the osmotic environments of the vertebrate groups (Table 1.1)

Ammonia is the nitrogenous waste product in teleosts and amphibian larvae which, because of its high solubility and small size rapidly diffuses through any surface in contact with water and is lost readily across the gills of teleosts and the integument of amphibian larvae. Together with its high toxicity, the high solubility of ammonia has precluded its use as the final nitrogenous waste product in terrestrial vertebrates, since large volumes of water would be lost in order to excrete a small amount.

Uric acid is less toxic and poorly soluble in water and is excreted in a semi-solid form by birds and reptiles. In its precipitated form uric acid does not contribute to the osmolality of urine or faeces and thus its excretion requires minimal water.

Urea is less toxic than ammonia or uric acid and requires a moderately small amount of water for its excretion. Mammals convert their nitrogenous waste to urea via the urea-ornithine cycle and excrete it via the urine, whereas the elasmobranchs retain urea for use as an osmotic agent within their body fluids (see section 2.1).

1.3 Hormones

Endocrine glands are ductless organs or tissues, located throughout the body, which secrete a variety of chemically distinct hormones. These glands, via their

Table 1.1

Table 1.1 Major Nitrogen Excretory Products In Various Animal Groups
(Modified from Schmidt Nielsen, 1986).

Table 1.1 Major Nitrogen Excretory Products in Various Animal Groups
(Modified from Schmidt-Nielsen, 1986).

Animals	End Product of Protein Metabolism	Habitat
Teleosts	Ammonia	Aquatic
Elasmobranchs	Urea	Aquatic
Crocodiles	Ammonia, Uric Acid	Semi-aquatic
Amphibians, larval	Ammonia	Aquatic
Amphibians, adult	Urea	Semi-aquatic
Mammals	Urea	Terrestrial
Turtles	Uric Acid	Terrestrial
Lizards	Uric Acid	Terrestrial
Snakes	Uric Acid	Terrestrial
Birds	Uric Acid	Terrestrial

secretions, control all aspects of vertebrate physiology and work in synergy with other regulatory systems, such as the nervous system, to produce appropriate and coordinated homeostatic responses. Hormones can be broadly classified, on the basis of their chemical nature, as lipophilic or hydrophilic hormones, the former being predominantly soluble in lipid and the latter predominantly soluble in water. Lipophilic hormones include the steroids, thyroxine and retinoic acid, whereas hydrophilic hormones include large polypeptides such as insulin, small peptides such as angiotensin II (AII) and small charged molecules such as adrenaline.

Hormones are secreted into the blood circulation, carried throughout the body to bind to specific receptors in "target" organs or tissues, where they exert their physiological effects. In addition, a large number of tissues secrete substances termed paracrine hormones, which act locally, influencing the activity of adjacent cells. Examples include neurotransmitters, neurohormones, chemical agents of the immune system such as interferons and interleukins, and nitrous oxide, the endothelium-derived relaxation factor (Änggård, 1990). Autocrine hormones influence the activity of the cells in which they were produced. Cultured cells often respond to growth factors they secrete and many tumour cells over-produce and release growth factors that stimulate inappropriate and unregulated growth of the tumour itself. In addition they can also act in a paracrine manner causing another tumour to form.

In many cases, hormone synthesis and release is controlled ultimately by higher brain centres, which integrate and respond to internal inputs from the body and external inputs from the environment. In some cases hormone synthesis and release are controlled directly by physiological stimuli. Once released into the blood a hormone may remain in "free" form or, in some cases be bound to a "carrier" protein. The binding characteristics of these proteins may be high affinity-low capacity, if the protein is present in low concentration, or low affinity-high capacity for abundant proteins such as albumin or lipoproteins. Binding proteins act as a reservoir of hormone, containing perhaps 95% of the hormone in equilibrium with a free hormone fraction. As molecules of free hormone are eliminated from the plasma, displacement of the hormone-protein binding equilibrium ensures that a similar number of hormone molecules dissociate, so maintaining the free hormone concentration of the plasma. Only the free hormone can react with receptors to elicit a response.

The majority of hormones are inactivated by degradation and are subsequently either excreted from the body, or have their components reused. The site of degradation is often the target tissue itself but the blood and specialised tissues, such as the liver, may also act as major degradation sites. In addition some hormones may be excreted by the kidneys in their active form.

When a steady state exists (i.e. the blood concentration of the hormone is constant) the amounts of hormone entering and leaving the blood must be equal. This

leads to the concept of metabolic clearance rate (MCR) which is defined as the volume of blood irreversibly "cleared" of hormone per unit time (Tait, Little, Tait and Flood, 1962). Thus:

$$MCR\ (ml/h) = BPR\ (\mu g/h) / C\ (\mu g/ml)$$

where BPR = blood production rate i.e the net amount of hormone entering the blood per unit time

C = plasma hormone concentration.

Based on the above principle, isotopic dilution techniques such as constant infusion, have been used experimentally to measure hormone secretory dynamics (see Section 3.8).

1.4 Receptors

The actions of hormones are mediated by receptors. Hormone receptors are protein macromolecules which bind hormones with great specificity and high affinity and transduce agonist activity through a series of reactions into altered target cell function. Receptors for hydrophilic hormones are located on the cell surface, whereas receptors for lipophilic hormones are located within the cell. There is one exception however, the progesterone receptor of Xenopus oocytes, which is located on the cell surface (Maller and Krebs, 1980).

The response of a specific cell or tissue to a hormone is dictated by the receptors it possesses, and by the intracellular reactions initiated by the binding of any one hormone to its receptor. Membrane bound receptors bind hydrophilic hormones and this is thought to cause a conformational change. This change leads to the generation

of an intracellular signal that alters the behaviour of the target cell. There are two general ways in which cell surface receptors generate intracellular signals. One is by activating a plasma membrane bound or intracellular enzyme which catalyses the production of an intracellular mediator. It is the change in the intracellular concentration of this mediator which transduces the signal. Alternatively cell surface receptors may open or close gated ion channels in the plasma membrane. In nerve or muscle cells, channel opening produces a small and transient ion influx that triggers an action potential which is transmitted throughout the membrane of the cell and propagated to adjacent cells and cell networks. However, many electrically inactive cells have receptors that are functionally linked to calcium channels in the plasma membrane. Ligand binding activates the receptor thereby opening the channels, allowing calcium ions to enter the cytosol and act as a second messenger.

Intracellular receptors bind lipophilic hormones and are located within the cell nucleus or cytosol. Unoccupied receptors cannot bind DNA but when bound to a ligand they become activated and tightly bind specific regions of DNA, inducing transcription and protein synthesis.

1.5 Intracellular Mechanisms

The lipid nature of all cell membranes has dictated the mechanisms by which the principal classes of hormones exert their actions.

1.5.1 Lipophilic Hormones

As a consequence of their lipophilic nature these hormones are usually transported to their target tissues via carrier proteins. Once delivered to their target and released by their carriers, steroid hormones diffuse across cell membranes and interact with receptor proteins in the nucleus or cytosol to form complexes which accumulate in the nucleus. There they bind to specific regulatory DNA sequences to modify the transcription rates of adjacent genes (Figure 1.1). These steroid receptor complexes may also affect the stability of specific mRNA's. The effects of lipophilic hormones typically last from several hours to several days.

1.5.2 Hydrophilic Hormones

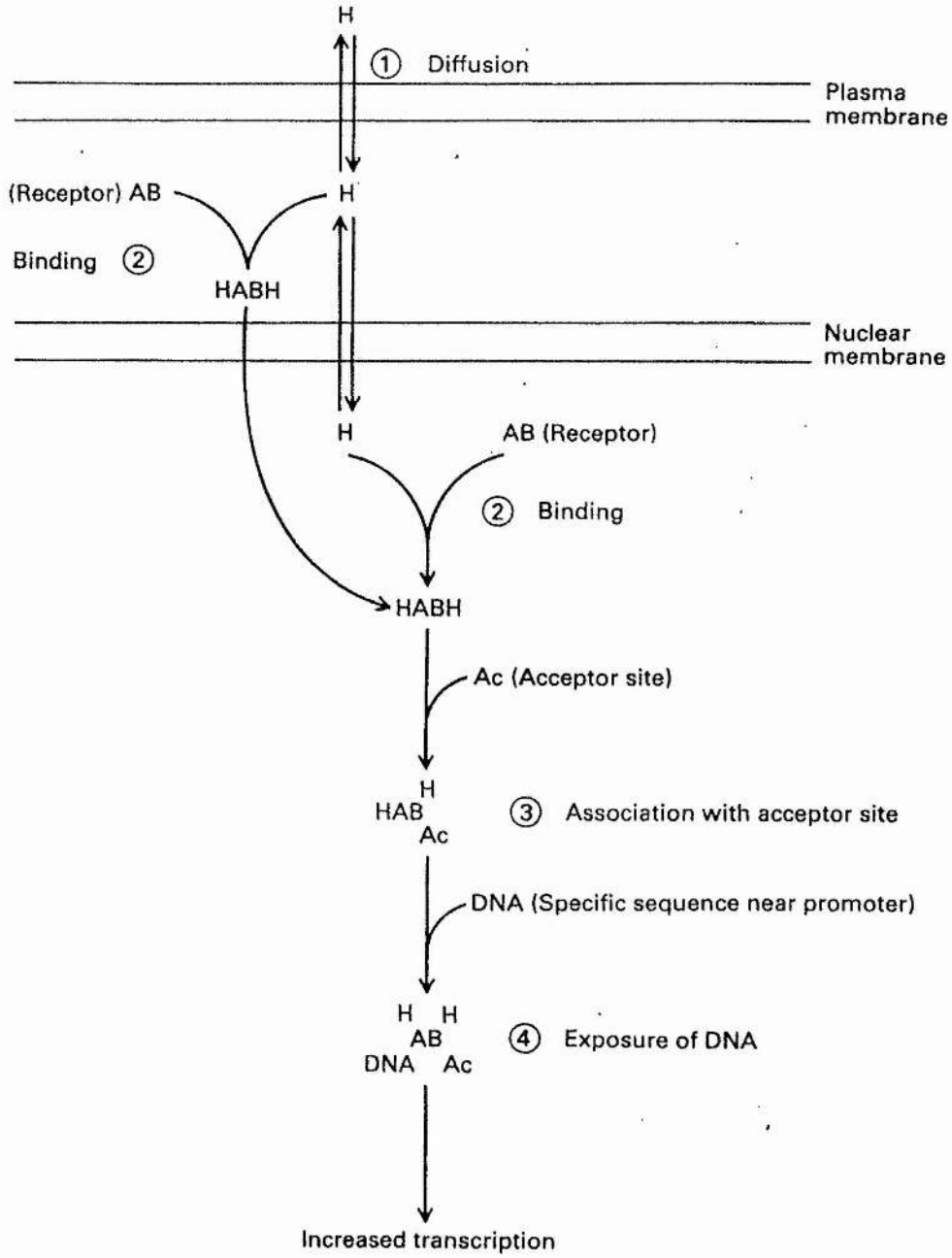
The majority of these hormones are not lipid soluble and cannot diffuse across the plasma membrane to interact with intracellular receptors. This large class of hormones interact with specific receptors on the cell surface and trigger the generation of intracellular messengers which elicit the desired response within the cell. Most surface-bound hormones exert their effects over a very short time period, although the responses to peptide growth factors may extend over several days.

1.5.2a Guanine Nucleotide-Binding Proteins

Guanine nucleotide-binding (G) proteins are a family of homologous membrane proteins which function in the transduction of plasma membrane receptor signals into intracellular effector responses. Twenty four G

Figure 1.1

Fig. 1.1 Model of Steroid Hormone Action
(From Martin, 1987).


H
① Diffusion
Plasma membrane
(Receptor) AB
H
Binding ②
HABH
Nuclear membrane
H
AB (Receptor)
② Binding
HABH
Ac (Acceptor site)
H
HAB
Ac
③ Association with acceptor site
DNA (Specific sequence near promoter)
H H
AB
DNA Ac
④ Exposure of DNA
Increased transcription

protein-coupled receptors have been cloned, and at least 86 molecularly or pharmacologically defined distinct receptors have been described. They are coupled to a large number of effectors by a family of up to 15 G proteins in mammalian cells and an as yet undisclosed number in non-mammalian cells (Birnbaumer et al., 1990).(Figure 1.2a).

G proteins consist of α,β and γ subunits and interconvert between an inactive GDP form and an active GTP form (Figure 1.2b). The exchange of GTP for bound GDP is catalysed by an activated receptor or hormone-receptor complex. Binding of GTP liberates the G_α subunit from the $G_{\beta\gamma}$ subunit dimer allowing G_α-GTP binding of the effector protein. G_α subunits also possess GTPase activity and will slowly hydrolyse bound GTP to GDP. Thus, the G protein has a built in deactivation device which limits the duration of its action on the effector protein.

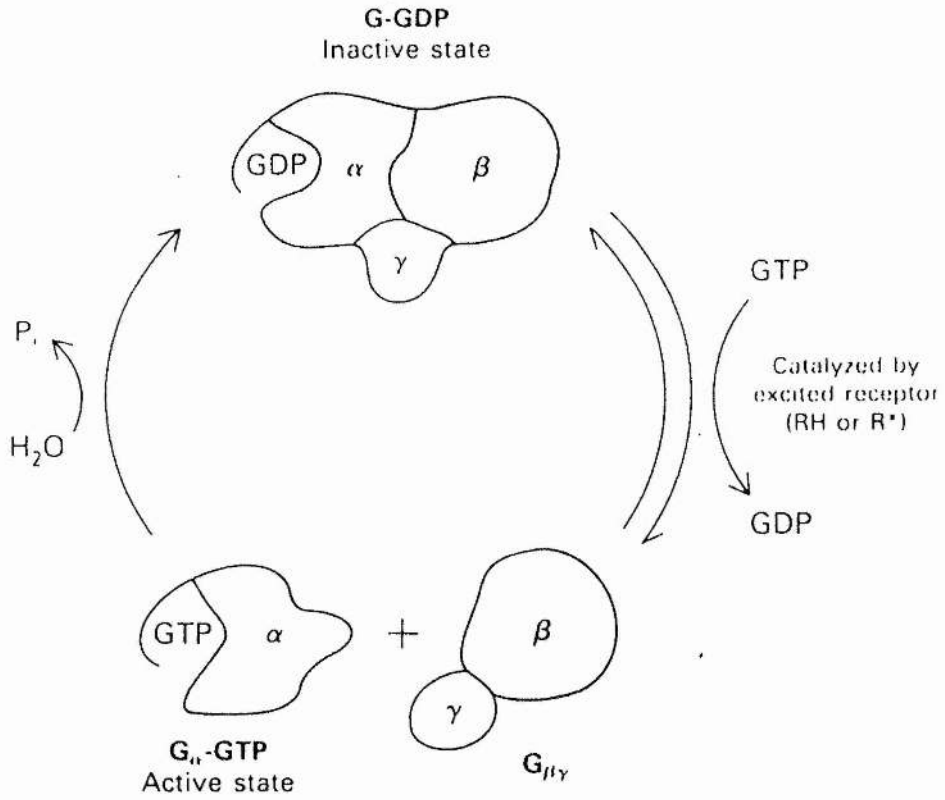
The proportion of G protein in the active GTP state depends on the rate of exchange of GTP for GDP compared with the rate of hydrolysis of bound GTP. In the absence of hormone the rate of GTP-GDP exchange is very low because the uncatalysed reaction is energetically unfavourable. As a consequence, nearly all of the G protein is in the inactive GDP form and the activity of the effector protein remains low. Hormone binding to a receptor leads to the amplified formation of G_α-GTP which binds and rapidly alters the activity of the effector protein. The hydrolysis of bound GTP by G_α closes this hormone triggered cycle (Stryer, 1988).

Figure 1.2

Fig. 1.2a Flow of Information Through G Protein-Dependent Signal Transduction Systems In Vertebrates (From Birnbaumer, 1990).

Fig. 1.2b Interconversion of G Proteins
G proteins interconvert between an inactive GDP form and an active GTP form. The exchange of GTP for bound GDP is catalysed by the hormone-receptor complex. G_{α}-GTP activates the effector protein. Hydrolysis of bound GTP brings the G protein back to the inactive state. The cycle is driven by the phosphoryl potential of GTP. (From Stryer, 1988).

	input	transduction			output
Location	Extracellular Milieu	Plasma Membrane			Intracellular Milieu or Plasma Membrane
		G-Protein Coupled Receptor		G-Protein Regulated Effector	
Functional Elements	Primary Messenger	→ R →	G Protein	→ E →	Secondary Messenger; Membrane Potential
Molecular Diversity					
# known	ca.60	87	12	> 15	cAMP, IP's, DAG, Ca^{2+}, cGMP, AA
# estimated	?	100-150	up to 15?	?	



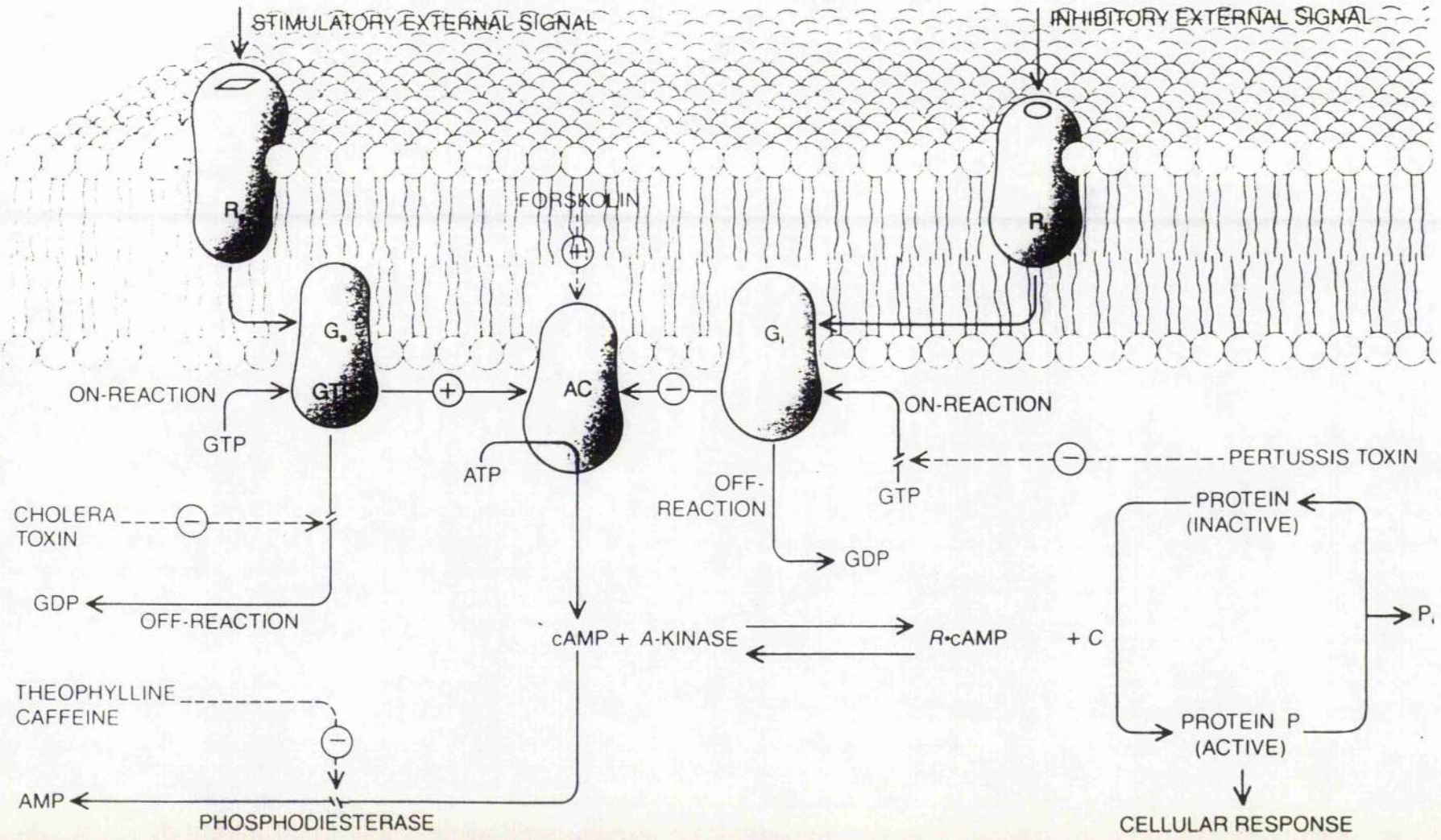
Several classes of G protein have been identified. Stimulatory G proteins (G_s) are involved in the stimulation of adenylate cyclase and also of dihydropyridine-sensitive Ca^{2+} channels. Many G_s are activated by cholera toxin. Inhibitory G proteins (G_i) are currently defined on the basis of their sensitivity to pertussis toxin, although initially they were classified on their ability to mediate hormonal inhibition of adenylate cyclase (Birnbaumer et al., 1990). G proteins that mediate the activation of phospholipases have not yet been biochemically identified. They are referred to as G_p and also, depending on whether the phospholipase is of the C or A2 type, as G_{plc} or G_{pla} (Birnbaumer et al., 1990). G_{plc} is involved in stimulation of the inositol-lipid signalling pathway whereas G_{pla} stimulates the release of arachidonic acid. G_t or transducin is an integral part of the visual cascade system and activates phosphodiesterase to greatly amplify the hydrolysis of cGMP in retinal rod cells, producing cell hyperpolarisation and signal generation in retinal neurons.

1.5.2b Adenylate Cyclase Cascade

Details of this signal pathway are given in Figure 1.3. Hormone binding to a stimulatory receptor results in the formation of an active hormone-receptor complex. This complex then activates a G_s which, in turn, transmits the signal to adenylate cyclase which is stimulated to produce cyclic AMP (cAMP) from ATP. Signal amplification occurs at both the activation of G_s and adenylate cyclase steps.

Figure 1.3

Fig. 1.3 Adenylate Cyclase Signal Pathway
Signals from stimulatory receptors (R_s) and inhibitory receptors (R_i) converge on the amplifier enzyme adenylate cyclase (AC), which converts ATP into cAMP. G proteins (G_s, G_i), which govern the convergence, are activated by GTP (on-reaction) and inactivated when GTP is hydrolysed (off-reaction) to GTP. cAMP binds to the regulatory component (R) of its protein kinase, liberating the catalytic component (C) which is then free to phosphorylate specific proteins that regulate a cellular response. Drugs affecting a particular stage in the sequence are indicated.
(From Berridge, 1985).


STIMULATORY EXTERNAL SIGNAL
INHIBITORY EXTERNAL SIGNAL
R_s
R_i
FORSKOLIN
G_s
GTP
G_i
ON-REACTION
AC
ON-REACTION
GTP
ATP
PERTUSSIS TOXIN
OFF-REACTION
GTP
CHOLERA TOXIN
GDP
GDP
OFF-REACTION
cAMP + A-KINASE
R•cAMP
+ C
PROTEIN (INACTIVE)
P_i
THEOPHYLLINE
CAFFEINE
PROTEIN P (ACTIVE)
AMP
PHOSPHODIESTERASE
CELLULAR RESPONSE

All of the known effects of cAMP in eukaryotic cells result from the activation of protein kinases (Stryer, 1988). In binding to the regulatory subunit of its protein kinase cAMP liberates the catalytic component which is then free to phosphorylate specific proteins that regulate a cellular response. In many cases, cAMP may also modulate the activity of another intracellular messenger, Ca^{2+}, which in turn governs the response. Cyclic AMP is inactivated, by a specific phosphodiesterase which hydrolyses it to AMP. Hormones known to operate via the adenylate cyclase cascade are listed in Table 1.2.

1.5.2c Guanylate Cyclase Pathway

The details of the guanylate cyclase-cyclic GMP (cGMP) pathway have not been as thoroughly investigated as those of the cAMP system. Hormone binding to a receptor results in stimulation of a membrane bound guanylate cyclase to produce cGMP. The cGMP then activates a cGMP-dependent protein kinase which acts to decrease intracellular Ca^{2+} concentration (Figure 1.4).

1.5.2d Inositol-Lipid Signalling Pathway

The binding of a hormone to its receptor triggers the activation of a G protein that activates phospholipase C (Figure 1.5a). This enzyme cleaves phosphatidylinositol 4,5-bisphosphate (PIP_2) to form inositol 1,4,5-trisphosphate (IP_3) and 1,2-diacylglycerol (DG) (Figure 1.5b). IP_3 diffuses through the cytosol to the endoplasmic reticulum (ER) where it binds to a receptor protein causing the release of Ca^{2+} from the ER into the cytosol. DG remains

Table 1.2

Table 1.2 Hormones Using cAMP as a Second Messenger

Table 1.2 Hormones Using cAMP as a Second Messenger

Adrenaline
Adrenocorticotropin
Calcitonin
Chorionic Gonadotropin
Follicle Stimulating Hormone
Glucagon
Luteinising Hormone
Lipotropin
Melanocyte-Stimulating Hormone
Noradrenaline
Parathyroid Hormone
Thyroid-Stimulating Hormone
Vasopressin

Figure 1.4

Fig. 1.4 The Guanylate Cyclase Signal Pathway

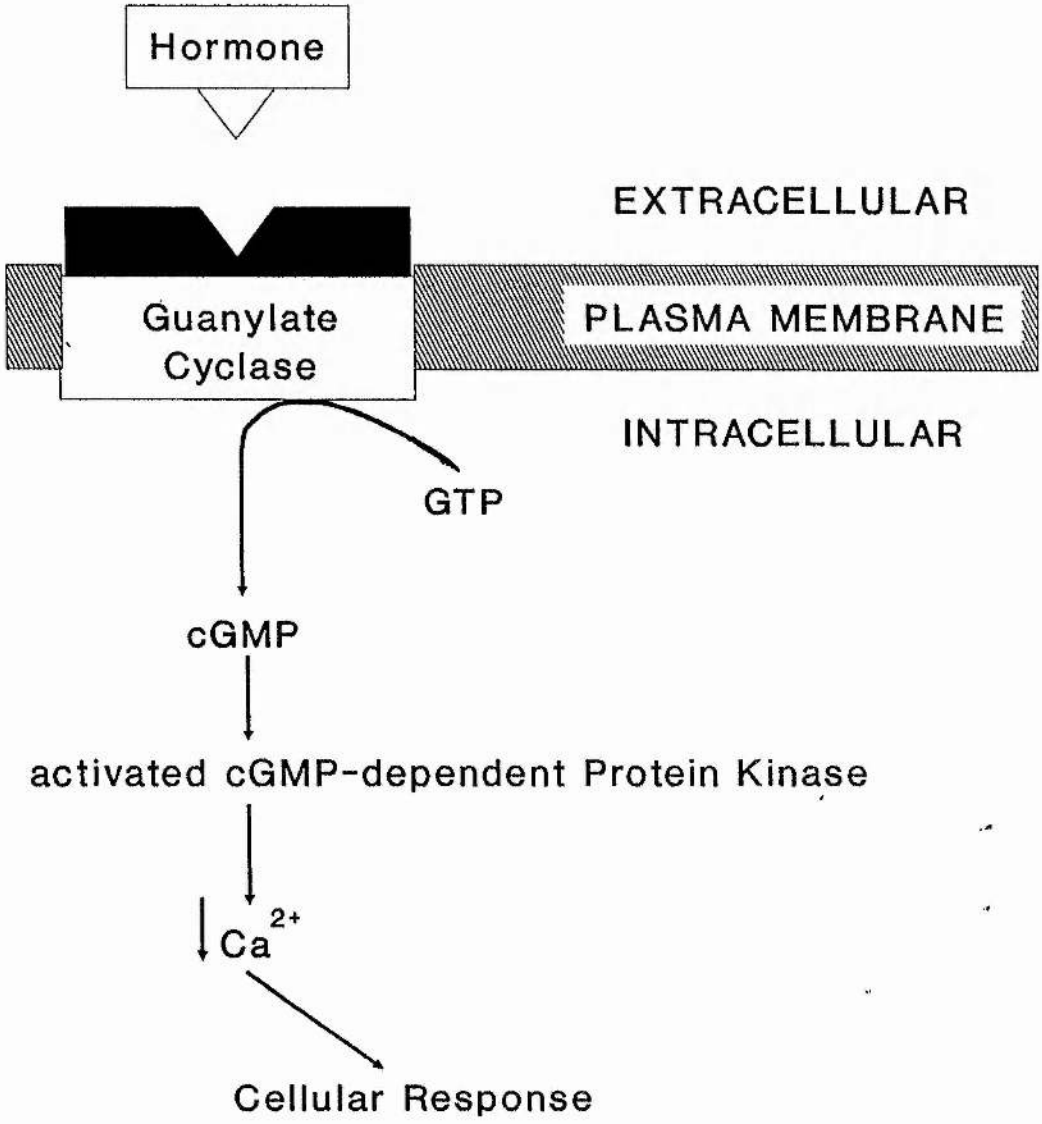
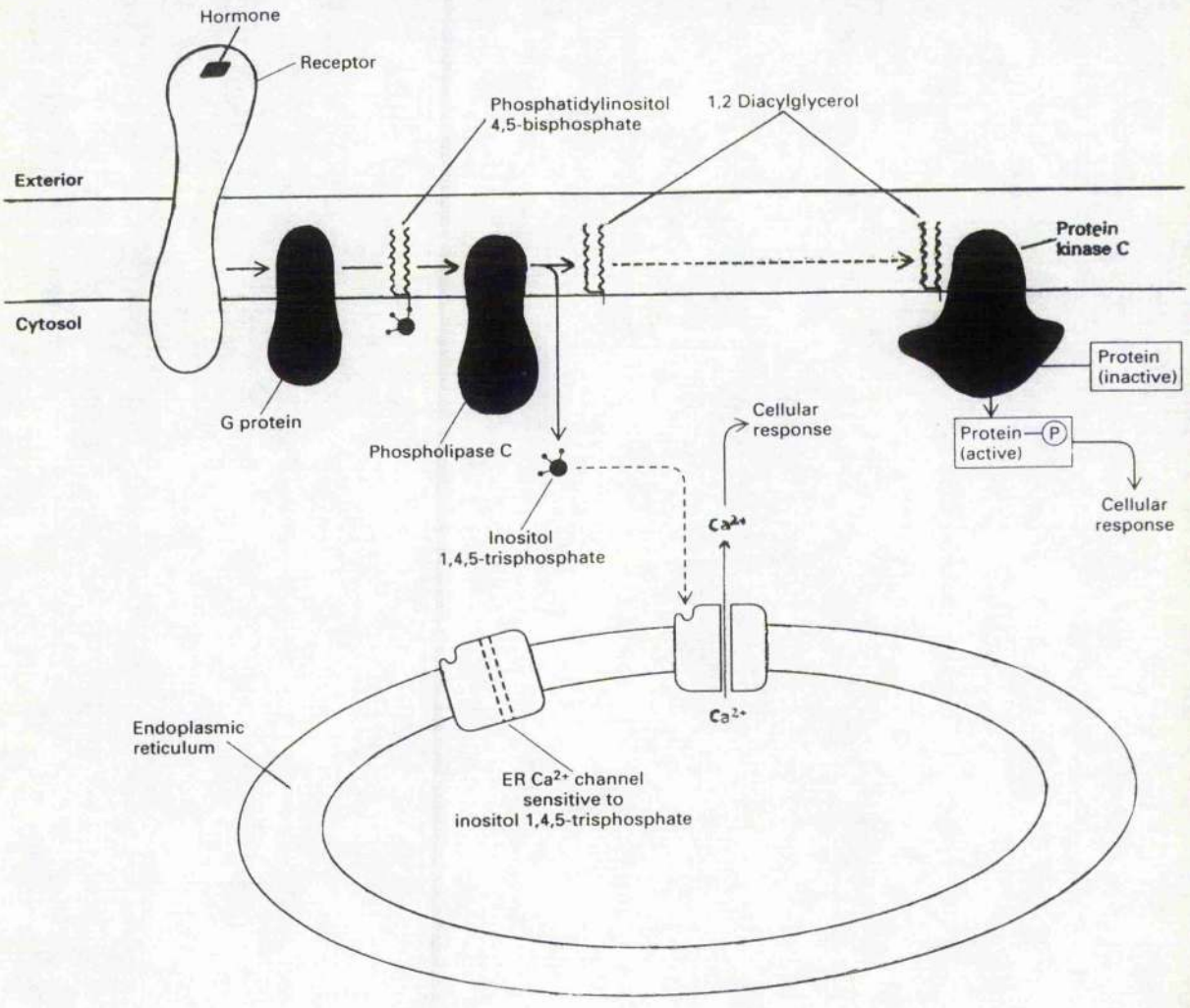
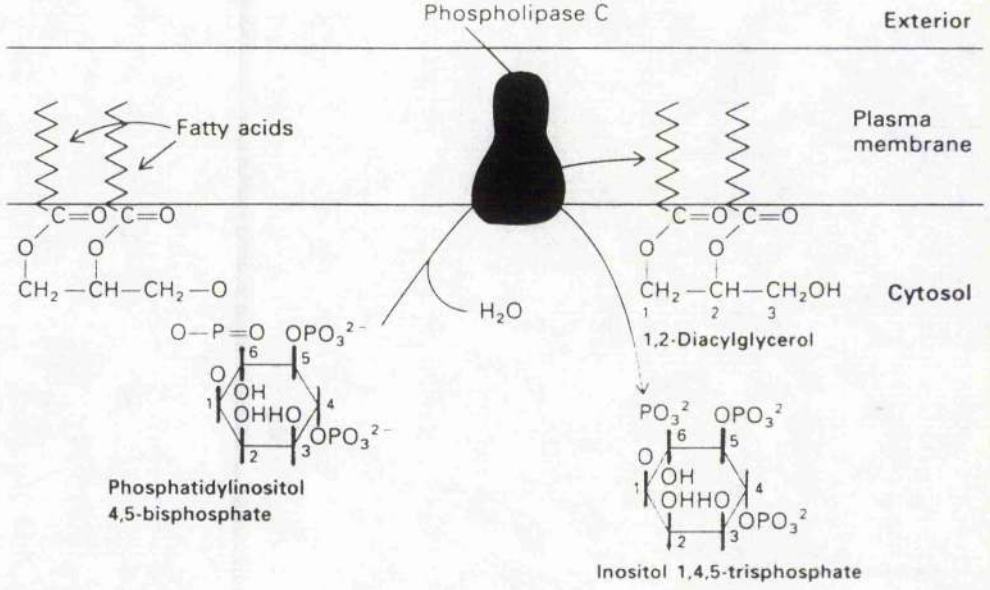

Hormone
EXTRACELLULAR
Guanylate Cyclase
PLASMA MEMBRANE
INTRACELLULAR
GTP
cGMP
activated cGMP-dependent Protein Kinase
Ca^{2+}
Cellular Response

Figure 1.5

Fig. 1.5a Second Messengers In the Inositol-Lipid Signalling Pathway
(From Darnell, Lodish and Baltimore, 1990).

Fig. 1.5b Hydrolysis of Plasma Membrane Phosphatidylinositol 4,5-bisphosphate
(From Darnell, Lodish and Baltimore, 1990).


Hormone
Receptor
Phosphatidylinositol 4,5-bisphosphate
1,2 Diacylglycerol
Exterior
Cytosol
Protein kinase C
Protein (inactive)
G protein
Phospholipase C
Cellular response
Protein (active)
P
Cellular response
Inositol 1,4,5-trisphosphate
Ca^{2+}
Ca^{2+}
Endoplasmic reticulum
ER Ca^{2+} channel sensitive to inositol 1,4,5-trisphosphate


Phospholipase C
Exterior
Fatty acids
Plasma membrane
C=O C=O
O O
CH_2—CH—CH_2—O
H_2O
C=O C=O
O O
CH_2—CH—CH_2OH
1 2 3
Cytosol
1,2-Diacylglycerol
O—P=O OPO_3^{2-}
6 5
O
1 OH 4
OHHO
2 3 OPO_3^{2-}
Phosphatidylinositol 4,5-bisphosphate
PO_3^{2} OPO_3^{2}
6 5
O
1 OH 4
OHHO
2 3 OPO_3^{2}
Inositol 1,4,5-trisphosphate

in the cell membrane where it increases the affinity of protein kinase C for Ca^{2+}, thereby rendering the enzyme active at physiological concentrations of this ion. Activated kinase C, in turn, phosphorylates several cellular enzymes and receptors, altering their activities to produce a cellular response (Stryer, 1988; Darnell, Lodish and Baltimore, 1990) (Figure 1.5a). Most of the effects of IP_3 and DG are synergistic.

IP_3 is short lived and within a second of its formation most is converted to inositol 1,4-bisphosphate, a molecule which cannot stimulate Ca^{2+} release. ER Ca^{2+} stores are finite and can be depleted within a few minutes. The maintenance of elevated cytosolic Ca^{2+} requires an influx of extracellular Ca^{2+} across the plasma membrane. In pancreatic cells it has been demonstrated that inositol 1,3,4,5-tetrakisphosphate, synthesised by the phosphorylation of IP_3, can mediate Ca^{2+} entry across the plasma membrane. In time this molecule is also inactivated by a phosphatase. In other cells evidence suggests that emptying of ER Ca^{2+} stores triggers extracellular Ca^{2+} influx via a mechanism other than the inositol phosphates (Darnell, Lodish and Baltimore, 1990).
IP_3 and DG are recycled to form phosphatidylinositol. In addition, DG can also be hydrolysed to glycerol and fatty acids. One of these fatty acids, arachidonic acid is the precursor of eicosanoid biosynthesis.

Arachidonic acid is released predominantly via the action of phospholipase A2 on phospholipids. The rate of

liberation of arachidonic acid is thought to be the rate limiting step for eicosanoid biosynthesis. Arachidonic acid is metabolised by three enzymes, mono-oxygenase, lipooxygenase, and cyclo-oxygenase, which give rise to the hydroxyeicosatetraenoic acids, the prostacyclins and the prostaglandins. The prostaglandins subsequently give rise to the thromboxanes. Prostaglandins have been the most studied of the eicosanoids and seem to modulate the action of hormones, often in the cells in which they were produced, as well as in adjacent cells (Martin, 1988: Stryer, 1988).

1.5.2e Calcium

Ca^{2+} is an intracellular messenger in many signal transducing pathways (see sections 1.5.2b,c above) and these systems along with other mechanisms regulate cytosolic Ca^{2+} concentration (Figure 1.6).

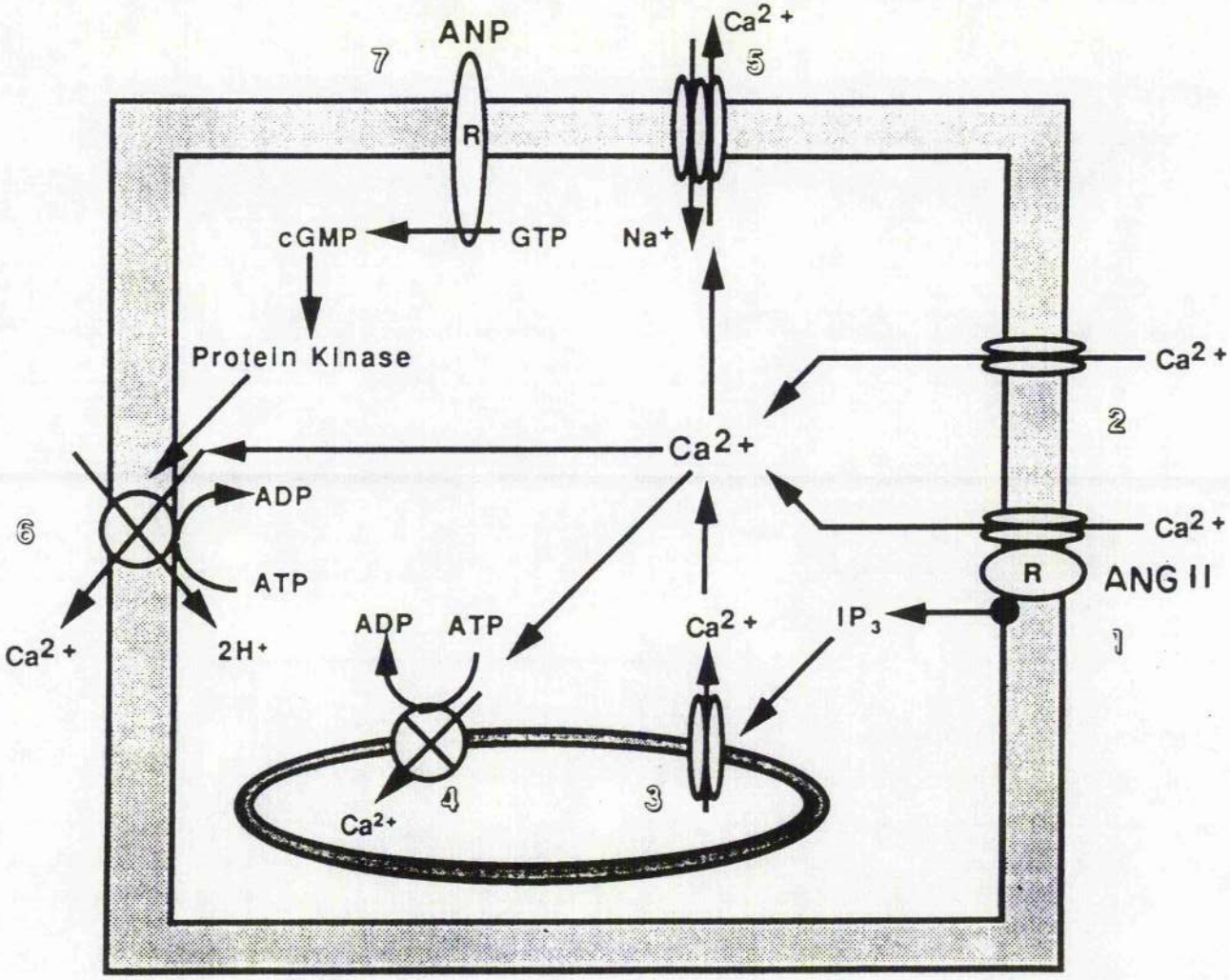
It is generally believed that two main properties of Ca^{2+} have led to its use as an intracellular messenger in nature. Firstly, intracellular Ca^{2+} concentration must be kept low because phosphate esters are abundant and calcium phosphates are insoluble. Intracellular Ca^{2+} concentration is typically several orders of magnitude lower than extracellular Ca^{2+} concentration and this gradient presents the cell with a potential means of mediating a signal: the cytosolic Ca^{2+} concentration can be abruptly raised for signalling purposes by the transient opening of Ca^{2+} channels in the plasma membrane or in an intracelluar membrane, such as that of the ER. Secondly, Ca^{2+} can bind

Figure 1.6

Fig. 1.6 Mechanisms of Transcellular and Intracellular Transport of Calcium

1. Receptor-gated Ca^{2+} channel
2. Voltage-sensitive Ca^{2+} channel
3. IP_3-gated calciosome Ca^{2+} channel
4. Calciosome Ca^{2+}-ATPase
5. Na^{+}-Ca^{2+} exchanger
6. Ca^{2+}-H^{+}-ATPase
7. ANP receptor-guanylate cyclase

ANG II, angiotensin II; IP_3, inositol triphosphate.
(From Brenner, Ballerman, Gunning and Zeidel, 1990).


ANP
7
R
Ca2 +
5
cGMP
GTP
Na+
Protein Kinase
Ca2 +
2
Ca2 +
6
ADP
ATP
2H+
Ca2 +
R
ANG II
IP3
1
ADP
ATP
Ca2 +
Ca2+
4
3

tightly to proteins. Because of its ability to form coordinate bonds to multiple oxygen atoms Ca^{2+} is able to cross-link different segments of a protein, inducing large conformational changes and altering protein activity. The binding of Ca^{2+} is highly selective and Ca^{2+} is preferred to Mg^{2+}, even when the latter is 1,000-fold more abundant (Stryer, 1988).

A large number of Ca^{2+}-sensing proteins exist, many possessing a common structural motif, the "EF hand", which forms a Ca^{2+}-binding site. The number of EF hands varies between different types of binding protein e.g. troponin C has four whereas parvalbumin from carp muscle has two.

An ubiquitous Ca^{2+}-binding protein, calmodulin, is responsible for mediating many of the intracellular responses to elevated cytosolic Ca^{2+} concentration. Calmodulin possesses four co-operative Ca^{2+}-binding sites, the binding of one Ca^{2+} facilitating the binding of the next until all the sites are occupied. In this way, small changes in cytosolic Ca^{2+} can produce large changes in calmodulin activity. Activated Ca^{2+}-calmodulin complexes stimulate a variety of proteins, including protein kinases, and produce a variety of coordinated cellular effects. One well studied enzyme affected by the Ca^{2+}-calmodulin complex is cAMP-dependent phosphodiesterase, which degrades cAMP and terminates its effects. Binding of the Ca^{2+}-calmodulin complex activates this enzyme and is one of many instances where Ca^{2+} and cAMP interact to finely tune aspects of cell regulation (Darnell, Lodish and Baltimore, 1990).

In summary, the calcium ion is an intracellular messenger involved in many aspects of cell control. Its cytosolic concentration is altered by many of the signal transduction pathways and in turn modulates the effects of many of these pathways. The calcium ion binds to a variety of proteins, directly influencing their activity and some of these proteins, such as calmodulin, can in turn activate other cellular proteins to produce a response.

1.6 Vertebrate Endocrinology

Endocrine glands have been identified among all of the vertebrate groups. Those common to the major groups include the pituitary, thyroid, endocrine pancreas, adrenal chromaffin and cortical tissue and gonads. Ultimobranchial bodies (or their homologue, thyroid "C-cells") are present in all vertebrates except the cyclostomes whereas the parathyroids are present in tetrapods but not in fishes. Teleost fish possess an additional endocrine organ, the Corpuscles of Stannius, and both teleost and elasmobranch fish possess a caudal neurosecretory system, named the urophysis. The following account will concentrate on those glands that have either a direct or an indirect effect on vertebrate osmoregulation.

1.6.1 Pituitary Gland

The morphology of the pituitary gland is similar in all vertebrates, being composed of two principal parts, the neurohypophysis (NHP) and the adenohypophysis (AHP). Both parts are derived from embryonic ectoderm, the NHP as a

downgrowth from the floor of the diencephalon and the AHP as an upgrowth from the roof of the mouth.

1.6.1a Adenohypophysis

The AHP synthesizes and secretes peptide hormones. These hormones fall into three categories: (1) the tropic hormones, growth hormone (GH) and prolactin; (2) the glycoprotein hormones, thyrotropin (TSH) and gonadotropin, of which there are two distinct forms in most vertebrates, follicle stimulating hormone (FSH) and luteinising hormone (LH) and (3) the corticomelanotropins, α- and β-melanotropin (MSH), adrenocorticotropin (ACTH) and β-lipotropin (LPH).

GH, prolactin, TSH, FSH and LH secretion occurs from distinct cells in the pars distalis, whereas ACTH is secreted by both the pars distalis and the pars intermedia, although the full molecule is normally only secreted from cells of the pars distalis. α-MSH, β-MSH and β-LPH are produced by cells of the pars intermedia by cleavage of the large precursor molecule, pro-opiocortin.

GH acts on carbohydrate and protein metabolism to stimulate body growth and may also have an osmoregulatory role in some vertebrates. The actions of prolactin vary within vertebrate groups and include effects on osmoregulation, reproduction and growth and development. ACTH influences corticosteroid secretion from adrenal or interrenal glands and TSH stimulates the thyroid gland. LH and FSH stimulate the gonads whereas MSH controls body colour change in many vertebrates.

1.6.1b Neurohypophysis

Neurohypophysial peptides are synthesized as prohormones by neurosecretory cells of the hypothalamus and are released from the terminals of long axons projecting into the NHP. All of the NHP peptides are comprised of 9 amino acids but distinct structural variations are evident between different vertebrate groups (Table 1.3). Cysteine residues at positions 1 and 6 are linked by a disulphide bond to form a cystine residue. In all the peptides characterised to date, the amino acids at positions 1, 5, 6, 7 and 9 are conserved, whereas those at positions 3,4 and 8 are variable (Acher, 1974, 1988; Acher and Chauvet, 1988).

The ten distinct NHP peptides identified from vertebrate subphyla can be divided into two groups: (1) basic peptides, which contain a basic amino acid (lysine or arginine) at position 8, and (2) neutral peptides, oxytocin-like peptides which contain neutral amino acids (leucine, isoleucine, valine or glutamine) at position 8 (Table 1.3).

Phenypressin, recently discovered in marsupials, is unusual in having a substitution at position 2, possessing phenylalanine rather than tyrosine. The elasmobranchs are also noteworthy, secreting several novel, neutral peptides in addition to the basic AVT.

In vertebrates the NHP peptides are associated with the regulation of water economy, and in mammals oxytocin is involved in the regulation of reproductive physiology.

Table 1.3

Table 1.3 Structure and Phylogeny of Vertebrate Neurohypophysial Hormones (From Batten and Ingleton, 1987).

Table 1.3 Structure and Phylogeny of Vertebrate Neurohypophysial Hormones

	Neutral Hormone	Basic Hormone
MAMMALS *Placentals* All except pigs	1 2 3 4 5 6 7 8 9 Cys Tyr-*Ile*-Gln-Asn-Cys-Pro-*Leu*-Gly-NH_2 Oxytocin	1 2 3 4 5 6 7 8 9 Cys-Tyr-*Phe*-Gln Asn Cys Pro-*Arg*-Gly NH_2 Arginine vasopressin
Domestic pigs	Oxytocin	1 2 3 4 5 6 7 Lys 9 Lysine vasopressin
Marsupials Macropodidae	Oxytocin	Lysine vasopressin + 1 Phe 3 4 5 6 7 8 9 Phenypressin
Phalangeridae	1 - 2 - 3 - 4 - 5 - 6 - 7 Ile 9 Mesotocin	Arginine vasopressin
BIRDS, REPTILES, AMPHIBIANS AND LUNGFISHES	Mesotocin	1 2 - Ile - 4 - 5 6 - 7 8 - 9 Arginine vasotocin
TELEOSTEAN AND GANOID FISHES	1 2 - 3 - Ser - 5 - 6 - 7 Ile 9 Isotocin	Arginine vasotocin
ELASMOBRANCHS *Selachii* Skates and Rays	1 2 - 3 - Ser - 5 - 6 - 7 - Gln 9 Glumitocin	Arginine vasotocin
Sharks and Dogfishes	1 - 2 - 3 - 4 - 5 - 6 - 7 Val - 9 Valitocin + 1 - 2 - 3 - Asn - 5 - 6 - 7 - 8 - 9 Aspar(g)tocin	Arginine vasotocin
Holocephali	Oxytocin	Arginine vasotocin
CYCLOSTOMES	------	Arginine vasotocin

1.6.2 Adrenocortical Homologue

1.6.2a Structure

As its name suggests, the adrenal gland is situated close to the kidneys, a situation that is ubiquitous throughout the vertebrates. In mammals, the gland is comprised of an inner medulla, made up of neural tissue, and an outer cortex, of mesodermal origin. The latter constitutes the steroid producing adrenocortical tissue while the former is the producer of catecholamines and is termed chromaffin tissue because of its dark staining properties in the presence of potassium dichromate. There is close association between the two tissue types throughout the vertebrates.

In birds, adrenocortical and chromaffin tissue intermingle to varying degrees, depending on the species, and there is no coalescence of chromaffin tissue to form a medulla. The glands lie at the anterior end of the kidneys often, wholly or partly covered by the gonads (Holmes and Phillips, 1976).

The adrenal gland of reptiles is a discrete encapsulated organ situated near the kidney, and displays varying degrees of intermingling of chromaffin and adrenocortical tissue, similar to that observed in birds (Lofts, 1978).

The amphibian adrenal gland is not a discrete gland separated from the kidney. It lies on the ventral surface of the kidney with chromaffin tissue intermingling with adrenocortical tissue (Hanke, 1978).

In teleosts, holosteans and the coelacanth, the two tissues form a diffuse organ at the anterior of the kidney, surrounding the posterior cardinal veins, while the dipnoans possess an even more dispersed structure (Chester-Jones and Mosley, 1980).

In elasmobranchs there is complete separation of chromaffin and adrenocortical tissues. The latter comprises a discrete gland, the interrenal gland, which is situated near the midline of the kidney, whereas the former is located in small islets running the length of the kidney, close to but distinct from the adrenocortical tissue. Cyclostome interrenal tissue has not been definitively identified. In lampreys (Petromyzonidae) it is assumed to lie along the walls of the cardinal veins whereas in hagfish (Myxinidae) it has not been positively identified (Chester-Jones and Mosley, 1980).

Zonation of adrenocortical tissue is evident in mammals but little or no zonation is observed in the glands of non-mammalian vertebrates. The ultrastructure of adrenocortical tissue is essentially similar throughout the vertebrates (Lofts and Bern, 1972). Characteristic features include large mitochondria, containing tubular christae and dense matrix, extensive smooth endoplasmic reticulum and numerous electron dense droplets, containing precursors of steroid synthesis such as cholesterol esters. Throughout the vertebrates the adrenocortical homologue secretes a discrete number of steroid hormones, known as the corticosteroids.

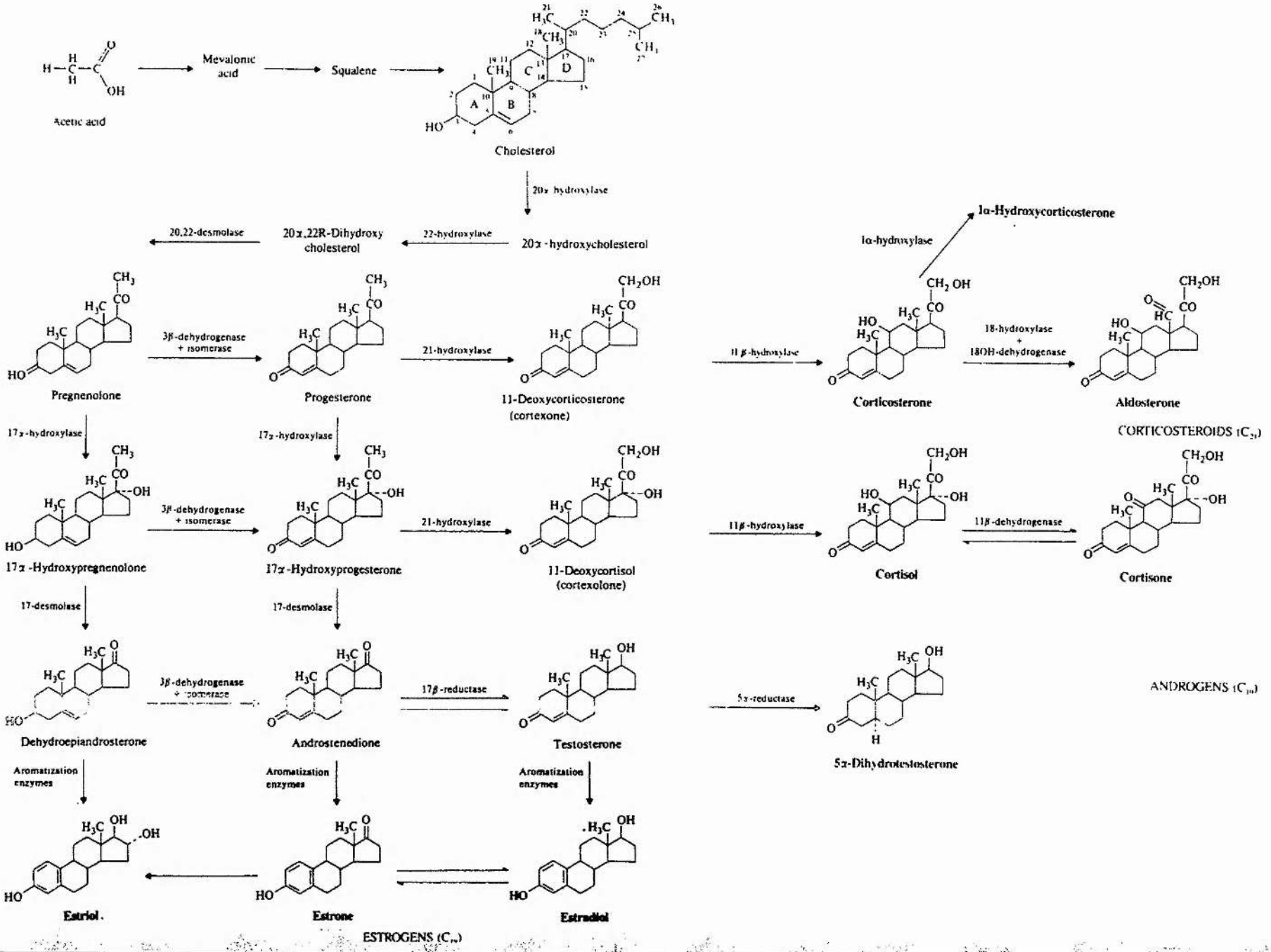
1.6.2b Corticosteroids

The steroids are lipid hormones with a basic structure consisting of four fused carbon rings, the so-called cyclopentanoperhydrophenanthrene nucleus. The variety of enzymes involved in steroid biosysnthesis produce an array of steroid hormones which can be classified into groups on the basis of the number of carbon atoms which they contain i.e. the oestrogens, containing 18 carbon atoms, the androgens, containing 19 carbon atoms and the corticosteroids, containing 21 carbon atoms. The oestrogens and androgens are known collectively as the sex-steroids because of their effects on reproductive processes and sexual characteristics. The corticosteroids primarily affect hydromineral balance and intermediary metabolism. This section will deal only with the corticosteroids.

The corticosteroids are secreted by adrenocortical tissue. The biosynthetic pathway of corticosteroid synthesis has been well documented in mammals and is generally true for non-mammalian vertebrates, although some species differences are evident. Figure 1.7 summarises the interrelationships and formation of the steroid hormones. Cholesterol is the precursor of all steroid biosynthesis. In mammals it is biosynthesised from acetic acid via a complex series of reactions, although it is equivocal whether all vertebrates synthesize cholesterol in this way (Sandor, Fazekas and Robinson, 1976). The first important stage in the formation of the corticosteroids is the conversion of cholesterol to pregnenolone. This

Figure 1.7

Fig. 1.7 Interrelationships and Formation of the Steroid Hormones
(Modified from Bentley, 1982).


Acetic acid
Mevalonic acid
Squalene
Cholesterol
20α-hydroxylase
20α-hydroxycholesterol
22-hydroxylase
20α,22R-Dihydroxy cholesterol
20,22-desmolase
Pregnenolone
3β-dehydrogenase + isomerase
Progesterone
21-hydroxylase
11-Deoxycorticosterone (cortexone)
17α-hydroxylase
17α-Hydroxypregnenolone
17α-Hydroxyprogesterone
11-Deoxycortisol (cortexolone)
17-desmolase
Dehydroepiandrosterone
Androstenedione
17β-reductase
Testosterone
Aromatization enzymes
Estriol
Estrone
Estradiol
ESTROGENS (C18)
11β-hydroxylase
18-hydroxylase + 18OH-dehydrogenase
Corticosterone
Aldosterone
1α-hydroxylase
1α-Hydroxycorticosterone
CORTICOSTEROIDS (C21)
11β-dehydrogenase
Cortisol
Cortisone
5α-reductase
5α-Dihydrotestosterone
ANDROGENS (C19)

sequence of reactions is controlled by a complex series of enzymes within the mitochondria and involves the cytochrome P-450 system. This conversion pathway is thought to be an important, rate-limiting step in corticosteroidogenesis (Burnstein, Kimball and Gut, 1970; Hochberg, MacDonald and Lieberman, 1973; Hochberg, Ladany and Lieberman, 1974). From pregnenolone the adrenocortical hormones are synthesized either via progesterone and 11-deoxycorticosterone into corticosterone and aldosterone, or via 17-hydroxyprogesterone and 11-deoxycortisol into cortisol (Schulster, Burnstein and Cooke, 1976; see Figure 1.7).

Corticosteroids are often divided into two structural categories, 17-hydroxycorticosteroids (cortisol, cortisone) and 17-deoxycorticosteroids (corticosterone, aldosterone). In most species one or the other predominates although some species may secrete different types in infancy and adulthood. Elasmobranchs are unusual in that their major interrenal secretion is the unique corticosteroid, 1α-hydroxycorticosterone (1α-OH-B) (Kime, 1977; Hazon and Henderson, 1984, 1985). First identified by Idler and Truscott (1966a), 1α-OH-B is formed by the 1α-hydroxylation of corticosterone (Figure 1.7). The enzyme catalysing this conversion, 1α-hydroxylase, is irreversibly inactivated at 37°C which may account for its absence in mammals. Table 1.4 lists the major corticosteroids produced by the vertebrate groups.

Table 1.4

Table 1.4 Major Corticosteroids Produced in Vertebrate Groups
(From Balment and Henderson, 1987).

Table 1.4 Major Corticosteroids Produced in Vertebrate Groups
(From Balment and Henderson, 1987).

Group	Corticosteroids in Blood
Elasmobranchs	**1α-hydroxycorticosterone**, corticosterone, 11-deoxycorticosterone, 11-deoxycortisol
Bony Fish	**Cortisol**, cortisone, 11-deoxycortisol, corticosterone
Lungfish	**Cortisol**, aldosterone, 11-deoxycortisol, 11-deoxycorticosterone, corticosterone
Amphibians	**Corticosterone**, 18-hydroxycorticosterone, aldosterone, 11-deoxycorticosterone, cortisol
Reptiles	**Corticosterone**, 18-hydroxycorticosterone, aldosterone
Birds	**Corticosterone**, aldosterone, 11-deoxycorticosterone
Mammals	**Cortisol/corticosterone**, aldosterone, cortisone, 18-hydroxycorticosterone, 11-deoxycorticosterone

* Bold steroids are the major secretory product.

1.6.2c Functions

On the basis of their pharmacological effects on electrolyte balance and intermediary metabolism in mammalian bioassays, the corticosteroids have been divided into mineralocorticoids and glucocorticoids. Since these divisions were based upon the roles of corticosteroids in mammals their extrapolation to non-mammalian systems is largely inappropriate.

In mammals aldosterone is considered the major osmoregulatory steroid, acting mainly to promote sodium reabsorption in a variety of target organs, including the kidney, sweat glands, salivary glands, urinary bladder, intestine and mammary glands. Cortisol and corticosterone have lesser effects on osmoregulation and are predominantly involved in the control of intermediary metabolism in this group.

In amphibians, aldosterone promotes sodium flux across the skin of saline adapted frogs (Maetz, Jard and Morel, 1958) and may also promote renal natriuresis, possibly as a secondary effect to the skin flux (Henderson, Edwards, Garland and Chester-Jones, 1972). Active sodium transport by the colon and urinary bladder are also stimulated by aldosterone (Crabbe and De Weer, 1964; Cofre and Crabbe, 1967) and in high doses glucocorticoid-like activity has been demonstrated (Hanke and Newman, 1972).

In birds and reptiles evidence for mineralocorticoid actions is scarce. Corticosteroids have been reported to both increase and decrease salt gland secretion in reptiles

(Holmes and McBean. 1964; Bradshaw, 1975), while in birds they increase nasal gland secretion and increase sodium ion reabsorption (Holmes and Phillips, 1976).

Research on the effects of corticosteroids in teleosts has been done almost exclusively on cortisol. It is generally accepted that this hormone is involved in salt and electrolyte balance, particularly in euryhaline species during migration between fresh and seawater when its effects closely interact with those of prolactin. Specific effects include control of renal electrolyte reabsorption, maintenance of glomerular filtration rate (GFR) and urine production rate, control of sodium fluxes at the gills and urinary bladder, and increased intestinal absorption of sodium, chloride and water. Cortisol is also important in intermediary metabolism in teleost fish, promoting gluconeogenesis (Stimpson, 1965) and regulating protein catabolism (Robertson et al., 1961; Robertson, Hane, Wexler and Rinfret, 1963; Storer, 1967).

1.6.2d Control of Corticosteroidogenesis-Extracelluar Stimulatory Factors

The control of adrenocortical function by a hypothalamic-pituitary axis has been demonstrated to varying degrees in all vertebrate groups (Batten and Ingleton, 1987). The components of this cascade are well documented for mammals and consist of the following: a corticotropin releasing hormone (CRH), produced by the hypothalamus, is transported down short axons in the median eminence and released into vessels of the hypophysial

portal system where it is transported to cells of the pars distalis in the adenohypophysis. These cells are then stimulated to release ACTH which in turn acts on adrenal glomerulosa cells to maintain steroid production, and adrenal fasciculata and reticularis cells to stimulate glucocorticoid production (Smelik and Vermes, 1980). Non-mammalian ACTH's have been identified and sequenced and CRH has been reported in amphibians, teleosts and elasmobranchs (Batten and Ingleton, 1987; Olivereau, Ollevier, Vandesande and Verdonck, 1984; Vallarino et. al., 1989a).

A considerable number of other factors have been implicated in the control of corticosteroidogenesis. Angiotensin II (AII) stimulates aldosterone secretion in mammals but a consistent role for the renin-angiotensin system throughout the vertebrates remains equivocal. In birds, AII is reported to have no effect on chicken adrenals in vitro (de Roos and de Roos, 1963; Rosenberg, Pines and Hurwitz, 1988) and in vivo, homologous renal extracts did not alter plasma corticosteroid levels (Taylor, Davis, Bretenbach and Harcroft, 1970).

In amphibians, homologous renal extracts and AII stimulated both aldosterone and corticosterone secretion (Johnston, Davis, Wright and Howard, 1967; Taylor, Davis and Braverman, 1972; Hanke and Maser, 1984), while other studies showed no effect with AII (Ulick and Feinholz, 1968).

In reptiles, homologous renal extracts elevated plasma corticosterone concentration in the turtle, Pseudemys

sueanniensis, but not in the crocodile, Caiman sclerops (Nothstine, Davis and de Roos, 1971).

In euryhaline teleosts confusion surrounds the steroidogenic role of the RAS. Injections of homologous renal extracts and AII elevated plasma cortisol levels in the freshwater eel, Anguilla anguilla (Henderson, Jotisankasa, Mosley and Oguri, 1976) but in vitro studies failed to elicit a response in the tilapia, Sarotherodon mossambicus (Hanke and Maser, 1984). The relationship between renin and cortisol in seawater and during change of environment is still contentious (Kenyon, McKeever, Oliver and Henderson, 1985; Nishimura, Sawyer and Nigrelli, 1976; Nishimura, Norton and Bumpus, 1978; Borriraja, Henderson and Chester-Jones, 1973).

Prostaglandins have been shown to increase corticosteroid output in vitro by mammalian (Spät and Jozan, 1975; Ellis et al., 1978), amphibian (Perroteau et al., 1984; Delarue et al, 1986) and teleost adrenocortical tissues (Gupta, Lahlou, Botella and Porthé-Nibelle, 1985). Prostaglandins may also have a role in the calcium-mediated stimulation of aldosterone secretion by AII (Perroteau et al., 1984; Delarue et al., 1986), although their importance has been questioned (Enyedi, Spät and Antoni, 1981). Urotensin I, produced by the urophysis in teleosts, has structural homology with mammalian CRH and displays corticotropin-releasing properties in teleost fish (Lederis et al., 1985; Fryer and Lederis, 1985; Woo et al., 1985).

It remains speculative whether an urophysial-pituitary-adrenal axis operates in aquatic vertebrates.

Other humoral agents which have a stimulatory effect on corticosteroidogensis include vasoactive intestinal polypeptide (VIP), serotonin and acetylcholine (Albano et al., 1974; Kawamura et al., 1985; Kojima, Kojima, Shibata and Ogata, 1986; Leboulenger et al., 1984). The mammalian neurohypophysial peptides, vasopressin and oxytocin, have been shown to increase both aldosterone and corticosterone secretion in rat adrenals in situ, but this was observed to be a largely vascular effect (Hinson, Vinson and Whitehouse, 1986).

Inhibitory Factors

The catecholamines, adrenaline and noradrenaline have not been shown to affect steroid biosynthesis. However dopamine, the direct precursor of adrenaline has been shown to inhibit the latter phase of AII-induced stimulation of aldosterone secretion in isolated bovine glomerulosa cells (MacKenna, Island, Nicholson and Liddle, 1979; Sequira and MacKenna, 1985; Carey, 1986).

Atrial natriuretic peptide (ANP) inhibits aldosterone secretion, both by reducing renin secretion from renal juxtaglomerular cells and by direct effects on the aldosterone secreting glomerulosa cells of the adrenal cortex. In the latter ANP inhibits basal, AII- and ACTH-stimulated aldosterone release and reduces aldosterone secretion in vivo (Brenner, Ballerman, Gunning and Zeidel, 1990). Inhibition of aldosterone secretion by ANP is

thought to take place at a step that involves the delivery of cholesterol to the inner mitochondrial membrane cytochrome P-450 complex (Butlen,Mistaoui and Morel, 1987; Chanderbhan et al., 1986). A second inhibitory site, of quantitatively lesser importance, is the conversion of corticosterone to aldosterone (Campbell, Currie and Needleman, 1985; Schiebinger et al., 1988), which is the main site of secretagogue stimulation in mammals. In birds ANP inhibits basal and ACTH-stimulated aldosterone secretion but has no effect on corticosterone secretion (Rosenberg, Pines and Hurwitz, 1988). In amphibians ANP inhibits both ACTH- and AII-stimulated corticosteroid secretion in vitro but has no apparent effect on basal secretion (Lihrmann et. al., 1988).

Electrolytes

The electrolyte composition of the extracellular fluid bathing adrenocortical cells is particularly important in the control of steroid secretion, both in vivo and in vitro. In mammals, adrenocortical cells have been shown to be very sensitive to potassium ions. Both aldosterone and corticosterone, but not cortisol, respond in a dose-dependent manner to small increases in potassium ion concentration (Haning, Tait and Tait, 1970; Muller, 1971). In birds, increases in potassium ion concentration had no effect on aldosterone secretion but inhibited corticosterone secretion (Rosenberg, Pines and Hurwitz, 1988). Doses of potassium ions, similar to those used in the mammalian studies, had no effect on amphibian steroid

production (Lihrmann et al., 1985) and stimulation was only achieved using very high doses (Masér, Janssens and Hanke, 1982). In the isolated perifused head kidney of the trout, Salmo gairdneri, increased potassium ion concentration produced no increase in cortisol output (Decourt and Lahlou, 1986).

While mammalian adrenocortical cells are sensitive to small increases in potassium ion concentration they are relatively insensitive to changes in sodium ion concentration, disproportionately large decreases being required to produce a modest elevation in hormone output (Muller, 1971). In amphibians, reductions in sodium ion concentration stimulate both corticosterone and aldosterone secretion in vitro (Maser et al., 1982; Crabbe, 1966) and in vivo (Davis, Copeland, Taylor and Baumber, 1970). In teleosts, only restoration of sodium concentration to its control level following a period of reduced concentration causes an increase in cortisol secretion (Decourt and Lahlou, 1986).

Extracellular calcium is important in maintaining basal steroidogenesis and in mediating ACTH-induced steroidogensis in both mammals (Foster, Lobo, Rasmussen and Marusic, 1981) and amphibians (Lihrmann et al., 1985). In vitro, bovine, rat and frog adrenocortical cells are stimulated in a dose-dependent manner by calcium (Neher and Milani, 1978; Yanagibashi, 1979; Lihrmann et al., 1985). Intracellular calcium is also involved in mediating steroid secretion and is discussed in the following section.

1.6.2e Control of Corticosteroidogenesis-Intracellular

ACTH operates via the adenylate cyclase system and the initial steps are well understood: ACTH binds its receptor and activates adenylate cyclase, via a stimulatory G protein, to produce cAMP. The cAMP then activates a cAMP-dependent protein kinase which, in turn, phosphorylates a number of intracellular proteins. Key targets of this kinase are ribosomal proteins which, once activated, stimulate the production of a specific factor, or factors, that directly catalyse the conversion of cholesterol to pregnenolone (Ferguson, 1963). The precise nature of the factor(s) is unclear but a small number of high and low molecular weight proteins have been sequenced and can directly affect mitochondrial steroidogenesis, possibly via increased binding of cholesterol to cytochrome P-450 (Vinson, 1987)

This is, however, an oversimplification as no close correlation between intracellular cAMP levels and the rate of aldosterone synthesis exists (Kojima, Kojima and Rasmussen, 1985c). ACTH also induces Ca^{2+} influx and it has been proposed that it acts on two receptor types: one, a high affinity-low capacity receptor, modulates Ca^{2+} influx and the other, a low affinity-high capacity receptor, modulates the adenylate cyclase cascade system (Kojima, Kojima and Rasmussen, 1985a). The increase in Ca^{2+} influx provides a positive sensitivity modulation of the cyclase system and also activates calmodulin-dependent enzymes leading to initiation of steroidogenesis. The rise

in cAMP content leads to activation of cAMP-dependent protein kinases and as a result of phosphorylation by this kinase, proteins involved in the Ca^{2+} messenger system become more sensitive to activation by calcium. The two messengers, Ca^{2+} and cAMP, act in synergy to initiate and sustain adrenocortical steroidogenesis in response to ACTH (Figure 1.8a).

Potassium ions act in a similar manner stimulating both Ca^{2+} influx, through voltage dependent Ca^{2+} channels, and cAMP generation following initial membrane depolarisation (Figure 1.8a). However, in K^+-induced aldosterone synthesis Ca^{2+} influx is large and cAMP production small while the opposite is true during ACTH stimulation (Kojima, Kojima and Rasmussen, 1985a). Two types of Ca^{2+} channel have been demonstrated in rat adrenal glomerulosa cells: transient channels may control the initial Ca^{2+} influx in response to an elevated extracellular K^+, whereas dihydropyridine-sensitive, long-lasting channels may control the sustained Ca^{2+} influx, which is necessary for sustained aldosterone secretion (Matsunaga et al, 1987).

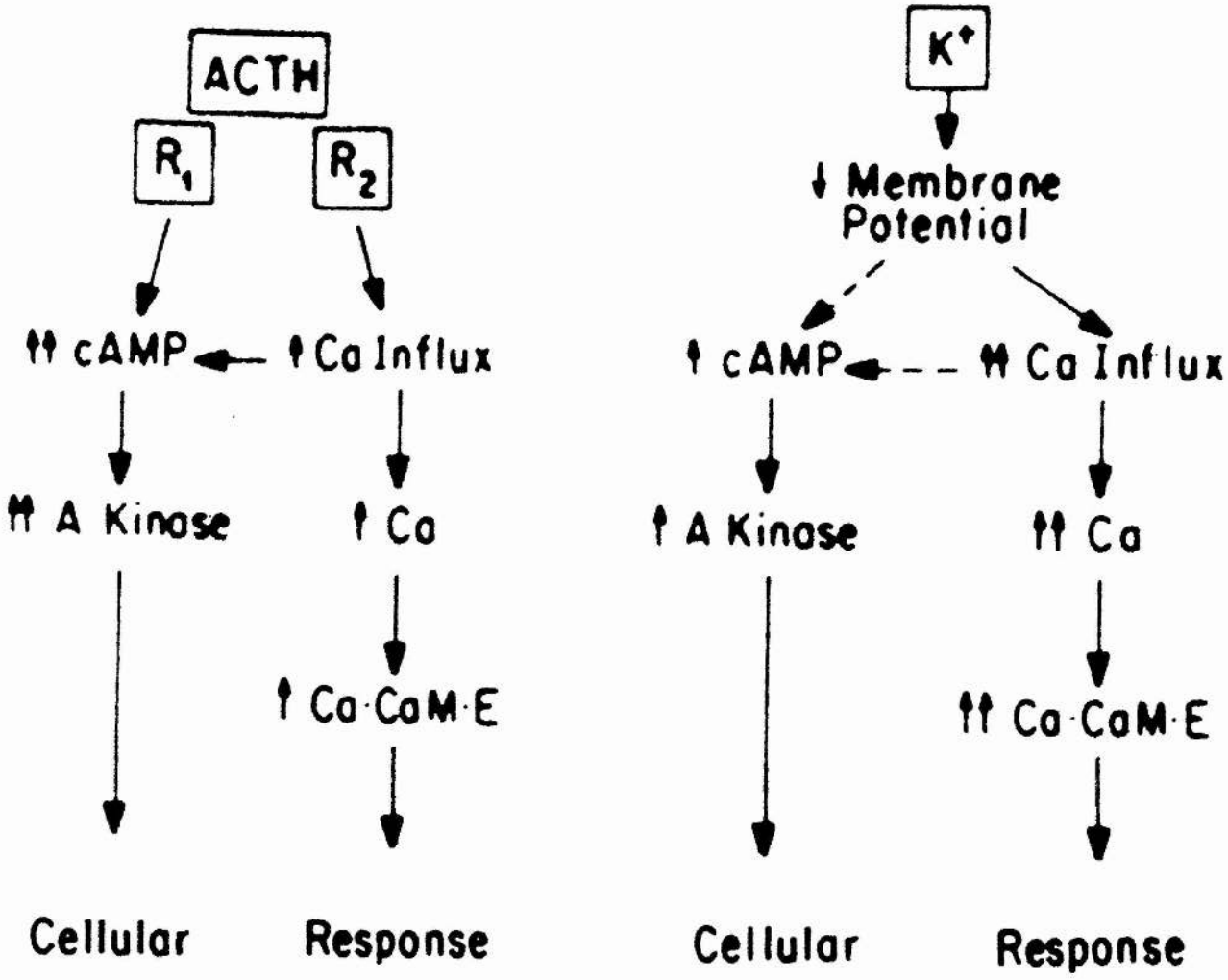
Angiotensin II produces a biphasic stimulation of aldosterone secretion in mammalian adrenocortical cells. Following AII receptor binding there are three immediate consequences: (a) an increase in cytosolic IP_3 concentration; (b) an increase in plasma membrane content of DG; (c) an increase in the rate of Ca^{2+} influx across the plasma membrane. The first effects result from the

Figure 1.8

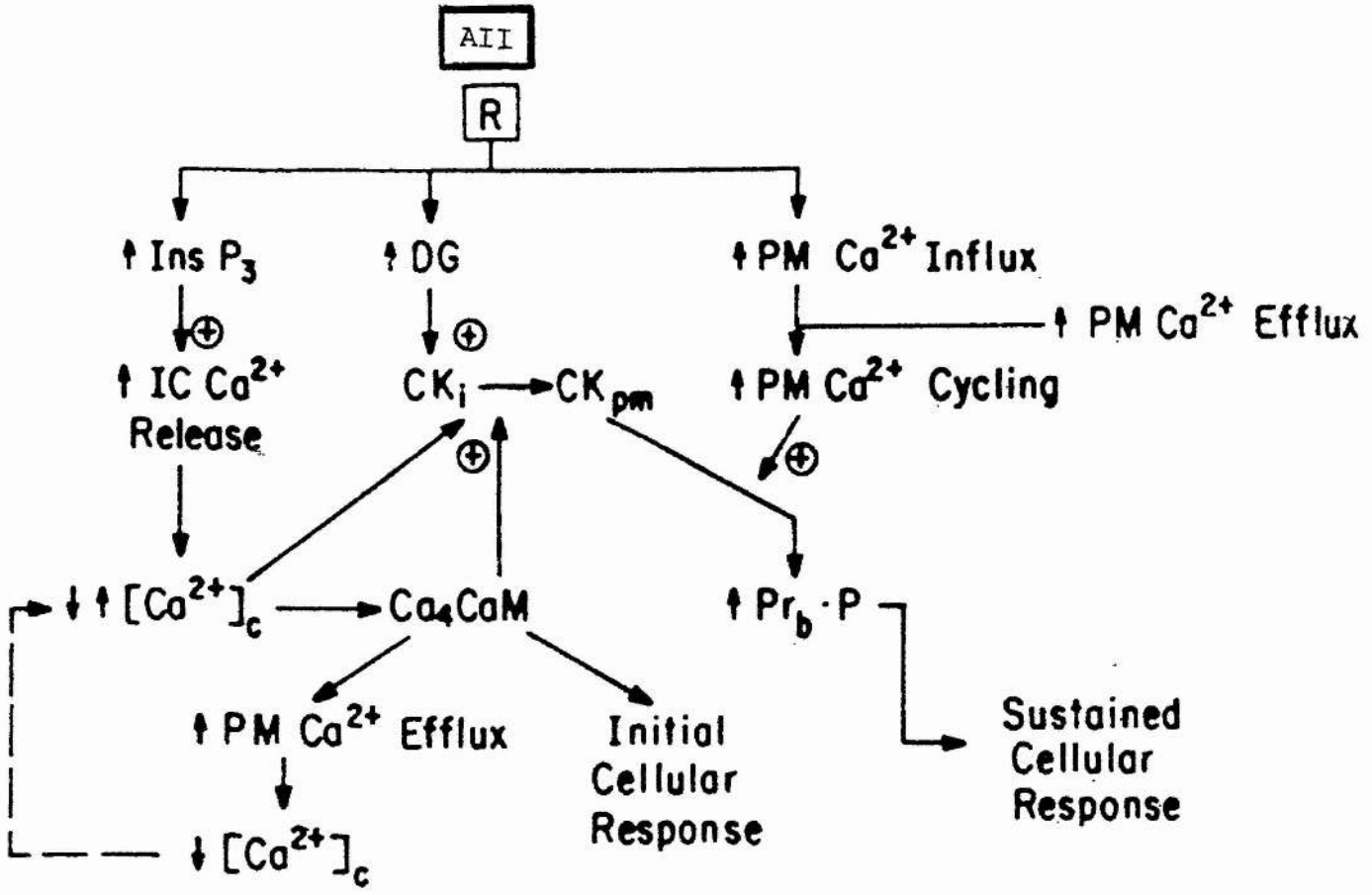
Fig. 1.8a Comparison of Mechanisms of ACTH- and Potassium-stimulated Corticosteroidogenesis
Ca CaM E, calmodulin-dependent enzymes.
(Modified from Kojima, Kojima and Rasmussen, 1985b).

Fig. 1.8b Mechanism of Angiotensin II stimulated Corticosteroidogenesis
Ins P_3, inositol 1, 4, 5-triphosphate; DG, diacylglycerol; PM, plasma membrane; IC Ca^{2+}, intracellular calcium; CK_i, inactive C-Kinase; CK_{pm}, active plasma membrane-bound C-Kinase; Pr_p P, phosphorylated cellular proteins.
(Modified from Kojima, Kojima and Rasmussen, 1985d).

a)


ACTH
R1
R2
↑↑ cAMP
↑ Ca Influx
↑↑ A Kinase
↑ Ca
↑ Ca·CaM·E
Cellular
Response
K+
↓ Membrane Potential
↑ cAMP
↑↑ Ca Influx
↑ A Kinase
↑↑ Ca
↑↑ Ca·CaM·E
Cellular
Response

b)


AII
R
↑ Ins P_3
↑ DG
↑ PM Ca^{2+} Influx
↑ PM Ca^{2+} Efflux
↑ IC Ca^{2+} Release
CK_i → CK_{pm}
↑ PM Ca^{2+} Cycling
↓ ↑ $[Ca^{2+}]_c$
Ca_4CaM
↑ $Pr_b \cdot P$
↑ PM Ca^{2+} Efflux
Initial Cellular Response
Sustained Cellular Response
↓ $[Ca^{2+}]_c$

activation of phospholipase C, which catalyses the hydrolysis of phosphatidylinositol 4, 5-bisphosphate (Kojima, Kojima and Rasmussen, 1985b), whereas the increased Ca^{2+} influx may result from G protein-stimulated activation of voltage-dependent Ca^{2+} channels in the plasma membrane (Kojima, Kojima, Shibata and Ogata, 1986). The increase in cytosolic IP_3 concentration induces the mobilisation of Ca^{2+} from a dantrolene-sensitive intracellular Ca^{2+} pool, presumed to be the endoplasmic reticulum, which leads to a transient rise in the Ca^{2+} concentration of the cell cytosol (Kojima, Kojima and Rasmussen, 1985b,c). This transient increase in cytosolic Ca^{2+} activates calmodulin-dependent enzymes, including calmodulin-dependent protein kinases, which initiate the aldosterone secretory response (Figure 1.8b). In addition, the rise in cytosolic Ca^{2+} concentration brings about the activation of another calmodulin-dependent enzyme, the Ca^{2+}/H^{+}-ATPase in the plasma membrane, which causes a net efflux of Ca^{2+} from the cell and a subsequent fall in cytosolic Ca^{2+} concentration (Kojima, Kojima and Rasmussen, 1985b).

The rise in DG together with the initial IP_3-stimulated increase in cytosolic Ca^{2+} (Figure 1.8b) cause the conversion of the Ca^{2+}-activated protein kinase (C-kinase) to its calmodulin-sensitive plasma membrane-bound form. Once activated this enzyme catalyses the phosphorylation of a specific subset of cellular proteins which, in turn, control the sustained

phase of aldosterone production (Figure 1.8b). The sustained presence of the activated C-kinase is dependent on the sustained presence of DG in the plasma membrane, while its turnover rate is controlled by the rate of calcium cycling across the membrane.

During the sustained phase of the response, the rates of plasma membrane Ca^{2+} influx and efflux are high i.e. the activated cell displays an increase in the rate of Ca^{2+} cycling across the plasma membrane without a net increase in total cell calcium (Figure 1.8b). The absence of a chronic increase in cytosolic Ca^{2+} is vitally important because the high concentration of phosphate esters present in the cell cytosol would react readily with the excess Ca^{2+} to produce insoluble calcium phosphates. The increased Ca^{2+} cycling and the activated C-kinase are thus functionally linked: the IP_3 stimulated transient rise in cytosolic free Ca^{2+} concentration, together with DG, initiates and controls the magnitude of activated C-kinase production while the rate of Ca^{2+} cycling across the plasma membrane governs the turnover rate of the activated enzyme.

The intracellular aspects of the control of corticosteroidogenesis have also been investigated, to a lesser extent, in non-mammalian vertebrates such as the frog, Rana ridibunda, and the teleost, Salmo gairdneri.

In amphibians the mechanisms of ACTH-stimulated and AII-stimulated aldosterone synthesis appear to be similar to those seen in mammals (Lihrmann et al, 1985, 1986, 1987). However, no stimulation was evident in response to

increased extracellular potassium concentration (Lihrmann et al, 1985), a result that was also observed in a marine teleost (Decourt and Lahlou, 1986). In the latter, as in mammals, the presence of extracellular Ca^{2+} is required for ACTH-induced corticosteroidogenesis.

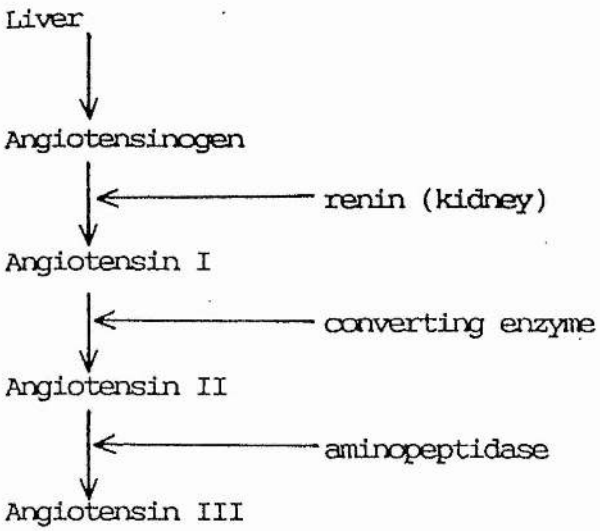
1.6.3 Renin-Angiotensin System

The renin-angiotensin system (RAS) is a diffuse hormonal system, with its peptide substrate and enzyme components located in the blood, and tissues such as liver, lung and kidney (Figure 1.9). In mammals, angiotensinogen, a large glycoprotein produced by the liver, is acted upon in the circulation by renin, a proteolytic enzyme synthesised and secreted by granulated renal juxtaglomerular cells, which cleaves off 10 amino acids to form the peptide angiotensin I (AI). This first step is rate limiting. Angiotensin converting enzyme (ACE) cleaves amino acids 9 and 10 from AI leaving the biologically active octapeptide angiotensin (AII). AII is then subsequently degraded by angiotensinase or converted by aminopeptidase to the heptapeptide, Angiotensin III (AIII), which is thought to have some biological activity at least in mammals (Freeman, Davis, Lohmeier and Spielman, 1977) (Figure 1.9).

The basic structure of AII is conserved throughout the vertebrate groups with interchange of asparagine (Asn) and aspartic acid (Asp) occurring at position 1 and valine (Val) and isoleucine (Ile) at position 5. The structure of

Figure 1.9

Fig. 1.9 The Mammalian Renin-Angiotensin System (From Balment and Henderson, 1987).



POSITIONS OF PEPTIDE CLEAVAGE

(Renin substrate)

	1	2	3	4	5	6	7	8	9	10	11	12
Angiotensinogen	Asp	Arg	Val	Tyr	Ile	His	Pro	Phe	His	Leu	Leu	Val

↑ renin

Angiotensin I: Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu

↑ converting enyme

Angiotensin II: Asp-Arg-Val-Tyr-Ile-His-Pro-Phe

↑ aminopeptidase

Angiotensin III: Arg-Val-Tyr-Ile-His-Pro-Phe

AI is more diverse with several amino acids occupying position 9 (Table 1.5).

Elements of the RAS have been identified in all vertebrate groups although controversy surrounds the cyclostomes and elasmobranchs (Nishimura et al, 1970; Henderson, Oliver, McKeever and Hazon, 1981; Hazon and Henderson, 1985; Hazon, Balment, Perrott and O'Toole, 1989).

In mammals, the RAS functions in the regulation of blood pressure and salt and water balance, via control of the cardiovascular system and influences on renal and adrenocortical function and may also play a central integrative role in body fluid homeostasis. In non-mammalian vertebrates the concept of such a role for AII is difficult to uphold at present due to lack of experimental information. AII produces a pressor response in all vertebrates. In mammals this is mostly due to a direct action on vascular smooth muscle, with smaller contributions from changes in blood volume and catecholamine secretion (Feldberg and Lewis, 1964; Stokland, Thorvaldson, Ilebekk and Kiil, 1982). In non-mammals the proportion of the AII-pressor response mediated by catecholamines is variable, arguably being 100% in elasmobranchs and cyclostomes (Opdyke and Holocombe, 1976; Opdyke, Carroll, Keller and Taylor, 1981) and birds (Nishimura, Nakamura, Sumner and Khosla, 1982; Nakamura, Nishimura and Khosla, 1982; Wilson, 1984), but less in reptiles (Zehr, Cupollé, 1981; Carroll and Opdyke, 1982),

Table 1.5

Table 1.5 Angiotensin I Amino Acid Sequences from Various Vertebrates
Three-letter amino acid abbreviations are given in Appendix 1.

Table 1.5 Angiotensin I Amino Acid Sequences from Various Vertebrates

	Amino Acid Sequence									
Common structure	1 -	2 Arg	3 Val	4 Tyr	5 -	6 His	7 Pro	8 Phe	9 -	10 Leu
Species variation										
Eel	Asp/Asn				Val				Gly	
Salmon	Asn/Asp				Val				Asn	
Goosefish	Asn				Val				His	
Bullfrog	Asp				Val				Asn	
Snake	Asp/Asn				Val				Tyr	
Fowl	Asp				Val				Ser	
Bovine, turtle	Asp				Val				His	
Human, pig, rabbit rat, dog, horse	Asp				Ile				His	

amphibians and teleosts (Nishimura, Norton and Bumpus, 1978) (see Table 1.6)

AII influences mammalian renal function through both aldosterone-dependent and aldosterone-independent mechanisms. Of the latter, the local, very high concentrations of AII generated within the kidney may alter urinary excretion directly through changes in glomerular filtration rate (GFR) or tubular electrolyte reabsorption and/or indirectly through altered patterns of intrarenal blood flow distribution. In non-mammalian vertebrates renal responses vary, since renal function is very sensitive to variations in glomerular filtration rate due to changes in systemic blood pressure. In these groups the wider systemic effects of AII may also affect renal excretory patterns (Balment and Henderson, 1987) (Table 1.7).

The dipsogenic effects of AII have been demonstrated in many mammalian (Fitzsimmons, 1969) and non-mammalian vertebrates (see Table 1.8) and appears to depend on the normal drinking habits of the species concerned and their environment (Blair-West, Gibson, McKinley and Nelson, 1983). For example, frogs, animals that do not normally drink, cannot be induced to imbibe even after large doses of AII (Hirano, Takei and Kobayashi, 1978). Similarly, reptiles from arid habitats do not respond to this peptide (Kobayashi, Uemura, Wada and Takei, 1979), while those from more temperate habitats do (Table 1.8). Exogenous AII stimulates drinking in euryhaline and brackish water

Table 1.6

Table 1.6 Vascular Effects of Angiotensin in Non-Mammalian Vertebrates

Summary of the effect of synthetic AII and homologous renal products on blood pressure in some representative non-mammalian vertebrates. RE, renal extract; RE + plasma, product of homologous renal extracts and plasma.

Table 1.6 Vascular Effects of Angiotensin in Non-Mammalian Vertebrates

Species	Test Substance	Response	α-Adrenergic Blockade	Reference
Agnatha				
Myxine glutinosa	Mammalian AII	Pressor	100% phentolamine	Carroll & Opdyke (1982)
Chondrichthyes				
Squalus acanthias	Goosefish AII	Pressor	100% phentolamine	Carroll (1981)
Squalus acanthias	Synthetic AI, AII	Pressor	100% phentolamine	Opdyke & Holcombe (1976)
Scyliorhinus canicula	Mammalian AII	Pressor	-	Hazon et al (1989)
Osteichthyes				
Neoceratodus forsteri	Synthetic AII	Pressor	-	Sawyer et al (1976)
Anguilla rostrata	RE + plasma, synthetic AI, AII	Pressor	30-40% phentolamine	Nishimura et al (1978)
Cyclopterus lumpus	AII	Pressor	10% phentolamine	Carroll & Opdyke (1982)
Salmo gairdneri	Synthetic AII	Pressor	-	Gray & Brown (1985)
Amphibia				
Rana catesbiana	AII	Pressor	40% phentolamine	Carroll & Opdyke (1982)
Rana catesbiana	RE + substrate	Pressor	-	Johnston et al (1967)
Reptilia				
Pseudemys swanniensis	RE, RE + plasma	Pressor	-	Nothstine et al (1971)
P. scripta elegans	Mammalian AII	Pressor	50% phenoxybenzamine	Zehr et al (1981)
P. scripta elegans	Mammalian AI, AII	Pressor	39-50% phentolamine 87% phenoxybenzamine	Stephens (1981)
Chrysemys scripta elegans	AII	Pressor	80% phentolamine	Carroll & Opdyke (1982)
Caiman sclerops	RE, RE + plasma	RE only Pressor	-	Nothstine et al (1971)
Ptyas koros	RE, AI + AII	Pressor	AII, 50% by phentolamine	Ho et al (1984)
Aves				
Gallus domesticus	RE + plasma	Pressor	-	Taylor et al (1970)
Gallus domesticus	AII	Pressor	100% phentolamine	Carroll & Opdyke (1982)
Gallus domesticus	AII	Pressor	100% phentolamine	Moore et al (1981)
Gallus gallus	Homol., AI, AII	Pressor	60-70% phenoxybenzamine	Nashimura et al (1982)
Columba oenas	RE + plasma	Pressor	-	Capelli et al (1970)
Anas platyrhyndros	AII	Pressor	-	Wilson & Butler (1983)

Table 1.7

Table 1.7 Renal Effects of Angiotensin in Non-Mammalian Vertebrates

Summary of the effects of synthetic angiotensin II (AII) and the converting enzyme inhibitor, captopril (CAPT) on renal function in some represntative non-mammalian vertebrates.
INF, infusion; INJ, injection; PD, pressor dose; NPD, non-pressor dose; N, natriuresis; D, diuresis; AN, anti-natriuresis; AD, anti-diuresis.
(Modified from O'Toole, 1987).

Table 1.7 Renal Effects Angiotensin in Non-Mammalian Vertebrates

Species	Experimental Conditions	Glomerular Effect	Tubular Effect	Reference
OSTEICHTHYES				
Teleostei				
(Aglomerular)				
Lophius americanus	AII INF	-	N + D	Churchill et al (1979)
Opsanus tau	AII INJ	-	no effect	Zucker & Nishimura (1981)
(Glomerular)				
Anguilla rostrata	AII INF	GFR↑, N + D	-	Nishimura et al (1976)
Salmo gairdneri (FW)	CAPT INF	GFR↑, D	-	Henderson et al (1980)
Salmo gairdneri (SW)	CAPT INF	GFR↑, D	-	Kenyon et al (1985)
Dipnoi				
Protopterus aethopius	AII INF	moderate D + N	-	Sawyer (1970)
Neoceratodus forsteri		D	-	Sawyer et al (1976)
AMPHIBIA				
Xenopus laevis	AII INF	GFR constant	D + N	Henderson & Edwards (1969)
Bufo parcnemis	AII		AN + AD	Coviello (1969)
Calyptocephallala	AII (NPD)	AD + AN	AD + AN	Pang et al (1977)
cardiverbeia	AII (PD)	D + N	D + N	Pang et al (1977)
REPTILIA				
Pseudemys scripta	AII INF (PD)	GFR↓	-	Brown et al (1983)
AVES				
Gallus domesticus	AII	-	D + N	Langford & Fallis (1966)

Table 1.8

Table 1.8 Dipsogenic Effects of Angiotensin in Non -Mammalian Vertebrates

Summary of the effects of synthetic angiotensins and the converting enzyme inhibitor, captopril (CAPT) on drinking behaviour in some representative non-mammalian vertebrates.
* indicates species capable of surviving in brackish water.
(Modified from O'Toole, 1987).

Table 1.8 Dipsogenic Effects of Angiotensin in Non -Mammalian Vertebrates

SPECIES	HABITAT	TREATMENT	RESPONSE	REFERENCE
ELASMOBRANCHII				
Scyliorhinus canicula	SW*	AII	drinking	Hazon et al (1989)
TELEOSTEI				
(Euryhaline species)				
Anguilla japonica	FW	AII (eel)	drinking	Takei et al (1979)
Anguilla japonica	SW	AII	drinking	Takei et al (1979)
Anguilla japonica	FW	pithed/AII	drinking	Takei et al (1979)
Platichthys flesus	FW	AI, AII	drinking	Carrick & Balment (1983)
Platichthys flesus	SW	AI, AII	drinking	Carrick & Balment (1983)
seven brackish water species	BW	AII	drinking	Kobayashi et al (1983)
Fundulus heteroclitus		AII		Malvin et al(1980)
(Freshwater species)				
Natropis cornutus	FW	AII	no drinking	Beasley et al (1986)
Cottus bairdi	FW	AII	no drinking	Beasley et al (1986)
Carassius auratus	FW	AII	no drinking	Beasley et al (1986)
Carassius auratus	FW*	AII	drinking	Kobayashi et al (1983)
20 freshwater species	FW	AII	no drinking	Kobayashi et al (1983)
(Seawater species)				
Pleuronectes americanus	SW	AII	drinking	Beasley et al (1986)
Myxocephalus octodecemspinosus	SW	AII	drinking	Beasley et al (1986)
13 species SW fish	SW	AII	no drinking	Kobayashi et al (1983)
Sillage japonica	SW*	AII	drinking	Kobayashi et al (1983)
Mugil cephalus	SW*	AII	drinking	Kobayashi et al (1983)
Glussogobius giuris fasciatopunctatus	SW*	AII	drinking	Kobayashi et al (1983)
AMPHIBIA				
Rana brevipoda	FW	AII	no drinking	Hirano et al (1978)
Rana brevipoda	FW	dehydration	no drinking	Hirano et al (1978)
Rana temporaria		CAPT INJ	drinking	Bolton & Henderson (1987)
REPTILIA				
Iguana iguana		AII	drinking	Fitzsimons & Kaufman (1979
Calotes versicolor		AII	no drinking	Kobayashi et al (1979)
Kinosternon subrubrum AII		AII	drinking	Kobayashi et al (1979)
Elaphe quadrivirigata		AII	drinking	Kobayashi et al (1979)
AVES				
Zunotrichia lecophrysgambelli		AII	drinking	Wada et al (1975)
Coturnix coturnix japonica		AII	drinking	Takei (1977a,b)
Gallus domesticus		AII	drinking	Snapir et al (1976)

teleosts (Kobayshi, Uemura, Wada and Takei, 1978; Hirano and Hasegawa, 1984; Carrick and Balment, 1983) but its function in stenohaline species is less clear. Kobayashi et al (1978) maintain that AII is not dipsogenic in stenohaline species, a view contested by Perrott and Balment (1985) and Beasley, Shier, Malvin and Smith (1986). Recent results have shown that AII is also dipsogenic in elasmobranchs (Hazon, Balment, Perrott and O' Toole, 1989) (Table 1.8).

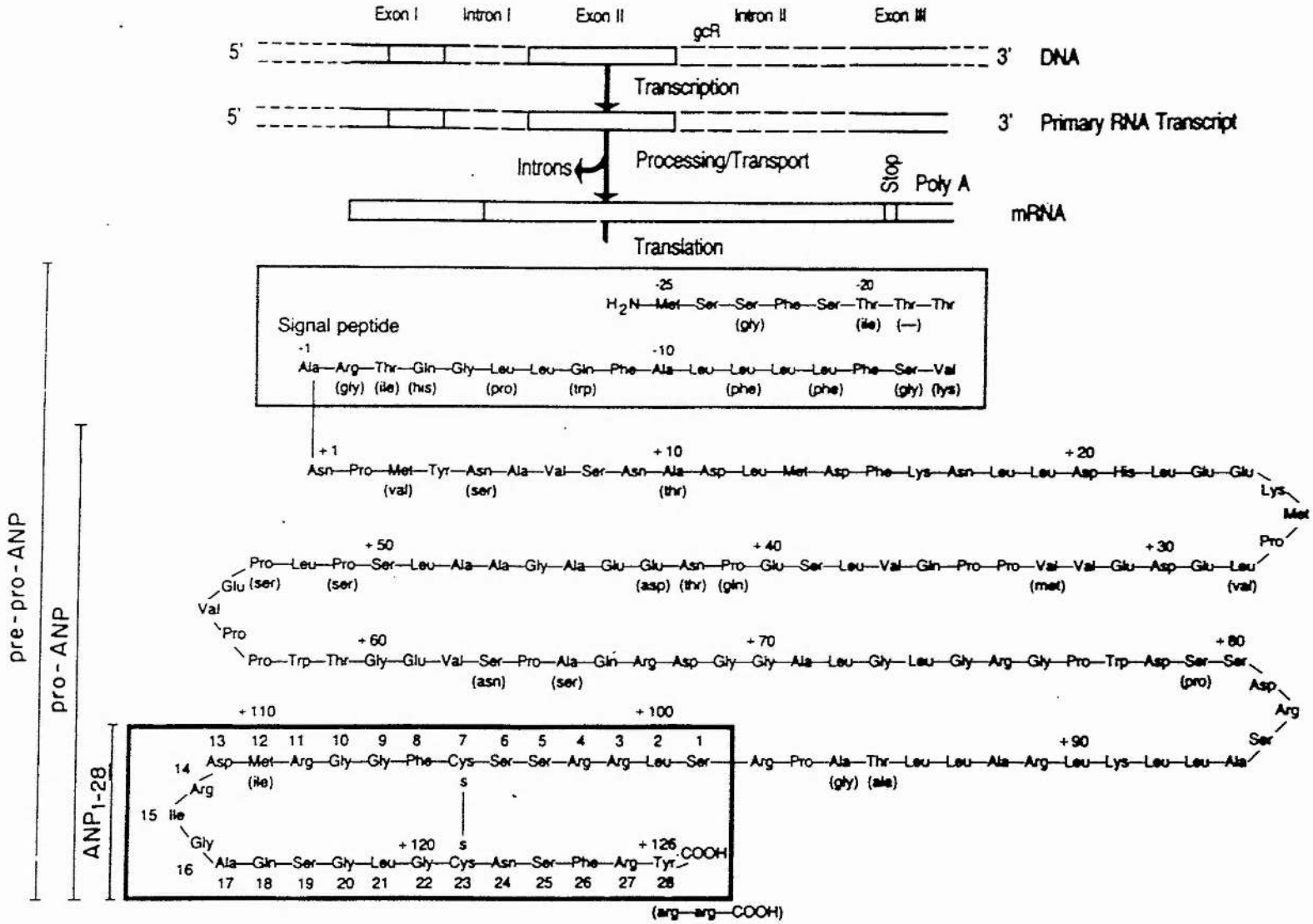
1.6.4 Atrial Natriuretic Peptide

In mammals atrial natriuretic peptide (ANP) is synthesized as a preprohormone in atrial myocytes and both the genes encoding preproANP and the amino acid sequence of this peptide show considerable homology between the species studied. In humans preproANP consists of 151 amino acids and cleavage of the amino-terminal "signal sequence" yields proANP-(1-126), the principal storage form of this peptide. Bioactive peptides are derived from the carboxy terminus and processing of proANP to ANP-(99-126), commonly known as ANP-(1-28), occurs immediately before and soon after secretion from the myocyte (Brenner, Ballerman, Gunning and Zeidel, 1990) (Figure 1.10). ANP-(1-28) is the predominant form entering carotid sinus blood.

The presence of a disulphide bond between cysteine residues at positions 7 and 23 is essential for ANP bioactivity: disruption of this structure by hydrolytic cleavage between residues 7 and 8 or residues 21-23 leads to almost complete loss of activity (Figure 1.10).

Figure 1.10

Fig. 1.10 Transcription and Translation of the Atrial Natriuretic Peptide Gene
(From Brenner, Ballerman, Gunning and Zeidel, 1990).


Exon I
Intron I
Exon II
gcR
Intron II
Exon III
5'
3'
DNA
Transcription
5'
3'
Primary RNA Transcript
Introns
Processing/Transport
Stop
Poly A
mRNA
Translation
Signal peptide
-25
-20
H2N—Met—Ser—Ser—Phe—Ser—Thr—Thr—Thr
(gly)
(ile) (—)
-1
-10
Ala—Arg—Thr—Gln—Gly—Leu—Leu—Gln—Phe—Ala—Leu—Leu—Leu—Leu—Phe—Ser—Val
(gly) (ile) (his)
(pro)
(trp)
(phe)
(phe)
(gly) (lys)
+1
+10
+20
Asn—Pro—Met—Tyr—Asn—Ala—Val—Ser—Asn—Ala—Asp—Leu—Met—Asp—Phe—Lys—Asn—Leu—Leu—Asp—His—Leu—Glu—Glu
(val)
(ser)
(thr)
Lys
Met
Pro
+50
+40
+30
Pro—Leu—Pro—Ser—Leu—Ala—Ala—Gly—Ala—Glu—Glu—Asn—Pro—Glu—Ser—Leu—Val—Gln—Pro—Pro—Val—Val—Glu—Asp—Glu—Leu
Glu (ser)
(ser)
(asp) (thr) (gln)
(met)
(val)
Val
Pro
+60
+70
+80
Pro—Trp—Thr—Gly—Glu—Val—Ser—Pro—Ala—Gln—Arg—Asp—Gly—Gly—Ala—Leu—Gly—Leu—Gly—Arg—Gly—Pro—Trp—Asp—Ser—Ser
(asn)
(ser)
(pro)
Asp
Arg
+110
+100
+90
13 12 11 10 9 8 7 6 5 4 3 2 1
Asp—Met—Arg—Gly—Gly—Phe—Cys—Ser—Ser—Arg—Arg—Leu—Ser
(ile)
S
Arg—Pro—Ala—Thr—Leu—Leu—Ala—Arg—Leu—Lys—Leu—Leu—Ala
(gly) (ala)
Ser
14
Arg
15
Ile
Gly
16
+120
+126
Ala—Gln—Ser—Gly—Leu—Gly—Cys—Asn—Ser—Phe—Arg—Tyr—COOH
17 18 19 20 21 22 23 24 25 26 27 28
(arg—arg—COOH)
pre-pro-ANP
pro-ANP
ANP1-28

Deletion of the 3 carboxy-terminal amino acids markedly reduces natriuretic and vasorelaxant activity (Brenner, Ballerman, Gunning and Zeidel, 1990). Extensions or deletions of the amino-terminus exert a less critical effect on bioactivity and even the storage form, proANP, has been shown to have ANP-like activity. The amino acid sequences of active vertebrate atrial natriuretic factors are given in Figure 1.11. A variety of other natriuretic peptides were purified and sequenced from atrial extracts by initial workers, but the majority of these have subsequently been shown to be artefacts of the extraction procedures used rather than naturally occurring forms of the peptide (Genest and Cantin, 1988; Brenner, Ballerman, Gunning and Zeidel, 1990) (Table 1.9).

ANP is released from atrial myocytes in response to atrial distension, resulting from increased intravascular volume. The combined effects of ANP on vasculature, kidneys and adrenals serve both acutely and chronically to decrease systemic blood pressure as well as intravascular volume. The reduction in blood pressure is the result of reduced peripheral vascular resistance (partly mediated by direct relaxation of vascular smooth muscle), diminished cardiac output and decreased blood volume. In the kidney ANP acts on specific receptors in the renal microvasculature and tubular epithelium to induce hyperfiltration, inhibition of sodium transport and suppression of renin release, all of which are responsible for increased natriuresis and diuresis and produce a

Figure 1.11

Fig. 1.11 Amino Acid Sequences of the Active Forms of Vertebrate Atrial Natriuretic Factors
Single letter amino acid abbreviations are given in Appendix 1.

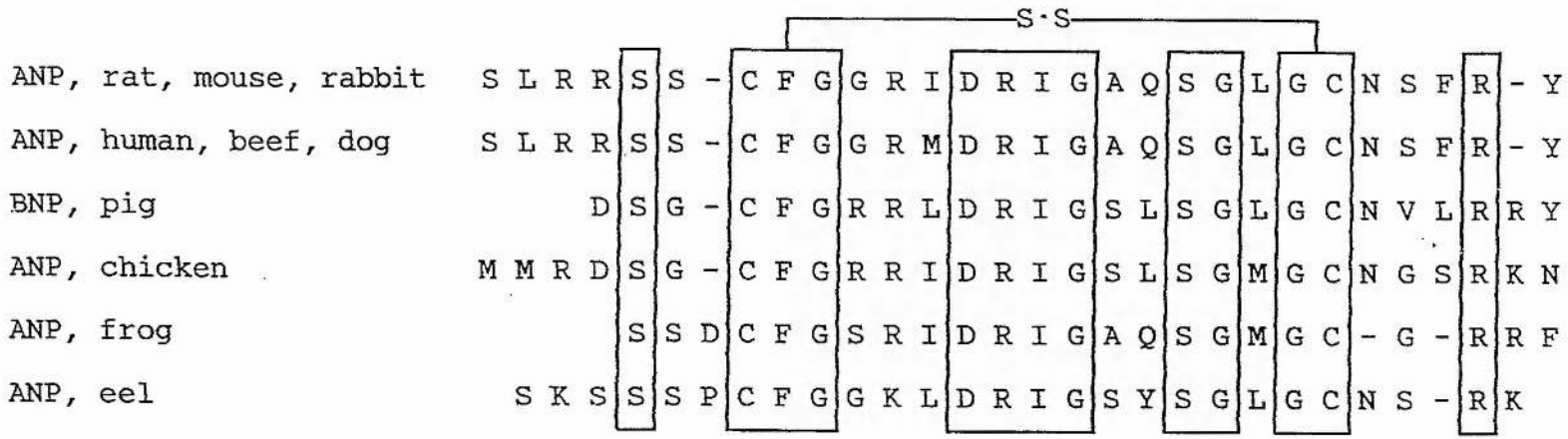

S·S
ANP, rat, mouse, rabbit S L R R S S - C F G G R I D R I G A Q S G L G C N S F R - Y
ANP, human, beef, dog S L R R S S - C F G G R M D R I G A Q S G L G C N S F R - Y
BNP, pig D S G - C F G R R L D R I G S L S G L G C N V L R R Y
ANP, chicken M M R D S G - C F G R R I D R I G S L S G M G C N G S R K N
ANP, frog S S D C F G S R I D R I G A Q S G M G C - G - R R F
ANP, eel S K S S S P C F G G K L D R I G S Y S G L G C N S - R K

Table 1.9

Table 1.9 Purification and Identification of Low Molecular Weight Forms of Atrial Natriuretic Peptide
(Modified from Genest and Cantin, 1988).

Table 1.9 Purification and identification of low molecular weight forms of atrial natriuretic peptide

Sequence	Common names	Peptide nomenclature	References
100 110 120 126			
S-L-R-R-S-S-C-F... I ...G-C-N-S-F-R-Y	Cardionatrin I	ANF (99 – 126)	Flynn et al. 1983
L-A-G-P-R-S-L-R-R-S-S-C-F... I ...G-C-N-S-F-R-Y	ANF (1 – 33)	ANF (94 – 126)	Thibault et al. 1983b
G-P-R-S-L-R-R-S-S-C-F... I ...G-C-N-S-F-R-Y	ANF (3 – 33)	ANF (96 – 126)	Seidah et al. 1984
R-R-S-S-C-F... I ...G-C-N-S-F-R-Y	ANF (8 – 33)	ANF (101 – 126)	
S-S-C-F... I ...G-C-N-S	Atriopeptin I	ANF (103 – 123)	
S-S-C-F... I ...G-C-N-S-F-R	Atriopeptin II	ANF (103 – 125)	
S-S-C-F... I ...G-C-N-S-F-R-Y	Atriopeptin III	ANF (103 – 126)	Currie et al. 1984b
S-C-F... I ...G-C-N-S	Des-Ser^{1}-Atriopeptin I	ANF (104 – 123)	Geller et al. 1984b
C-F... I ...G-C-N-S	Des-Ser^{1}-Ser^{2}-Atriopeptin I	ANF (105 – 123)	
S-S-C-F... I ...G-C-N	Des-Ser^{21}-Atriopeptin I	ANF (103 – 122)	
R-S-S-C-F... I ...G-C-N-S-F-R	Auriculin A	ANF (102 – 125)	Atlas et al. 1984
R-S-S-C-F... I ...G-C-N-S-F-R-Y	Auriculin B	ANF (102 – 126)	
A-L-L-A-G-P-R-S-L-R-R-S-S-C-F... I ...G-C-N-S-F-R-Y	ANF-I	ANF (92 – 126)	
G-P-R-S-L-R-R-S-S-C-F... I ...G-C-N-S-F-R-Y	ANF-II	ANF (96 – 126)	Misono et al. 1984a
G-P-R-S-L-R-R-S-S-C-F... I ...G-C-N-S-F-R	ANF-III	ANF (96 – 125)	Misono et al. 1984b
R-S-S-C-F... I ...G-C-N-S-F-R-Y	ANF-IV	ANF (102 – 126)	
A-L-L-A-G-P-R-S-L-R-R-S-S-C-F... I ...G-C-N-S-F-R-Y	β-rANP (14 – 48)	ANF (92 – 126)	
L-A-G-P-R-S-L-R-R-S-S-C-F... I ...G-C-N-S-F-R-Y	β-rANP (16 – 48)	ANF (94 – 126)	
A-G-P-R-S-L-R-R-S-S-C-F... I ...G-C-N-S-F-R-Y	β-rANP (17 – 48)	ANF (95 – 126)	
G-P-R-S-L-R-R-S-S-C-F... I ...G-C-N-S-F-R-Y	β-rANP (18 – 48)	ANF (96 – 126)	Kangawa et al. 1984a
S-L-R-R-S-S-C-F... I ...G-C-N-S-F-R-Y	α-rANP	ANF (99 – 126)	
R-S-S-C-F... I ...G-C-N-S-F-R-Y	α-rANP (4 – 28)	ANF (102 – 126)	
S-S-C-F... I ...G-C-N-S-F-R	α-rANP (5 – 27)	ANF (103 – 125)	
G-P-R-S-L-R-R-S-S-C-F... I ...G-C-N-S-F-R-Y	ANF A	ANF (96 – 126)	
A-G-P-R-S-L-R-R-S-S-C-F... I ...G-C-N-S-F-R-Y	ANF B	ANF (95 – 126)	Napier et al. 1984a
L-A-G-P-R-S-L-R-R-S-S-C-F... I ...G-C-N-S-F-R-Y	ANF C	ANF (94 – 126)	
S-L-R-R-S-S-C-F...M...G-C-N-S-F-R-Y	α-hANP	[Met^{110}]ANF (99 – 126)	Kangawa and Matsuo 1984
S-L-R-R-S-S-C-F...M...G-C-N-S-F-R-Y	human ANF	[Met^{110}]ANF (99 – 126)	Thibault et al. 1984a
S-L-R-R-S-S-C-F...M...G-C-N-S-F-R-Y	bovine ANF	[Met^{110}]ANF (99 – 126)	Ong et al. 1986

subsequent decrease in blood volume and blood pressure. ANP also acts to lower blood volume and blood pressure by inhibiting aldosterone secretion, both directly by acting on adrenocortical cells and indirectly by inhibiting renin secretion (Genest and Cantin, 1988; Brenner, Ballerman, Gunning and Zeidel, 1990).

Studies on ANP in non-mammalian vertebrates are less extensive. Amphibian and eel ANP's have been sequenced and binding sites for mammalian ANP have been demonstrated in the heart, renal system and aorta of the hagfish Mxyine glutinosa (Kloas, Flugge, Fuchs and Stolte, 1988) and ANP-like immunoreactivity has been detected in members of the agnatha, chondrichthyes and osteichthyes. ANP is thought to be involved in salt rather than water balance in fishes (Evans, 1990). Table 1.10 lists some of the reported effects of ANP in elasmobranchs and teleosts.

Table 1.10

Table 1.10 Effects of Atrial Natriuretic Peptide in Fishes

Summary of the effects of atrial natriuretic peptides and homologous heart extracts in some elasmobranchs and teleosts.
AP, atriopeptin; ANP, atrial natriuretic peptide; HE, homologous heart extract; GFR, glomerular filtration rate; UFR, urine flow rate.

Table 1.10 Effects of Atrial Natriuretic Peptide in Fishes

Species	Test Substance	Response	Reference
ELASMOBRANCHII			
Squalus acanthias	APII	Vasodepressor	Solomon et al (1985b)
Scyliorhinus canicula	human ANP	Vasodepressor	Hazon et al (1987)
Squalus acanthias	APII	Vasodepressor	Benyajati & Yokota (1990)
Squalus acanthias	APII	Vasodilation, arterial rings	Solomon, Solomon et al (1985)
Squalus acanthias	mammalian AP	Vasodilation arterial rings	Evans & Weingarten (1989)
Squalus acanthias	APII	GFR & UFR ⇔ 100% SW	Yokota & Benyajati (1986)
Squalus acanthias	APII	GFR & UFR ↓ 100% SW	Benyajati & Yokota (1990)
Squalus acanthias	APIII	GFR & UFR ↑ 90% SW	Benyajati & Yokota (1988)
Squalus acanthias	APII	GFR & UFR ↑ 70% SW	Solomon et al (1988)
Squalus acanthias	APII, HE	Rectal gland secretion ↑	Solomon et al (1985b)
Squalus acanthias	ANPII, HE	Rectal gland secretion ↑	Silva et al (1987)
Scyliorhinus canicula	human ANP	1α-OH-B secretion ↑	Hazon et al (1987)
TELEOSTEI			
Salmo gairdneri (FW)	synthetic ANP, HE	Vasopressor	Duff & Olson (1986)
Opsanus tau	APIII, HE	Vasodepressor (HE only)	Lee & Malvin (1987)
Salmo gairdneri (FW)	human ANP	↓ Pulse pressure	Eddy et al (1990)
Salmo gairdneri (FW)	rat ANP	Vasodilation, arterial rings	Olson & Meisheri (1989)
Opsanus beta	rat ANP	Vasodilation, arterial rings	Evans et al (1989)
Salmo gairdneri (FW)	synthetic ANP, HE	Diuresis, natriuresis	Duff & Olson (1986)
Opsanus tau	APIII, HE	Diuresis, natriuresis	Lee & Malvin (1987)
Pseudopleuronectes americanus	API, APIII	↓ Intestinal Na absorption	O'Grady et al (1985)
Fundulus heteroclitus	rat ANP	↑ Opercular Cl secretion	Scheide & Zadunaisky (1988)

2. ELASMOBRANCHII

2. Elasmobranchii

The Elasmobranchii are a subclass of the Chondrichthyes or cartilaginous fish and consist of two orders: the Selachii, sharks and dogfish, contain about 250 species and the Batoidei, skates and rays, contain about 350 species. The second Chondrichthyean subclass, the Holocephali, include the Chimaeroidea or ratfishes and rabbitfishes and only about 25 species have been identified. Historically these orders are of great antiquity first appearing on the geological time scale about 300 million years ago.

Common characteristics of the Chondrichthyes include a completely cartilaginous skeleton, unusual endocrine profiles and the retention of large amounts of urea. This "physiological uraemia" is closely regulated and is in stark contrast to the situation found in most other vertebrates, where urea is rapidly excreted as a nitrogenous waste product of protein catabolism.

Little work has been done on the Holocephali and the following sections will deal almost exclusively with the Elasmobranchii.

2.1 Elasmobranch osmoregulation

The definitive studies on elasmobranch osmoregulation were carried out by Homer Smith (1936, 1953) and culminated with his theory of "physiological uraemia". It was known that elasmobranchs possessed elevated body fluid osmolalities (Duval and Portier, 1923; Smith, 1931), had high levels of plasma and tissue urea (Stadeler and

Frerichs, 1858; von Schroeder, 1890), and did not drink seawater (Smith, 1931). This information, together with evidence that the kidneys of the spiny dogfish, Squalus acanthias, filtered molecules such as xylose, fructose and inulin (Clarke and Smith, 1932; Shannon, 1934 a,b) led Smith to postulate that marine elasmobranch fish avoided problems of osmotic water loss by maintaining plasma osmolality at a level higher than that of the surrounding medium. This hyperosmolality was predominantly due to the retention of large amounts of urea and trimethylamine oxide (TMAO), which were normally nitrogenous waste products in other vertebrates. As a consequence, the gills and skin of these fish were highly impermeable to these compounds (Duval, 1925) while the kidney retained up to 90% of the filtered urea and TMAO. These classical studies by Smith have formed the basis for many of the contemporary studies of elasmobranch osmoregulation.

Elasmobranchs adapt to varying environmental salinities by altering plasma osmolality, maintaining it slightly hyperosmotic to the environment. The changes in plasma osmolality are achieved by varying plasma concentrations of urea, methylamines and electrolytes. In detailed studies on the lesser spotted dogfish, Scyliorhinus canicula, Hazon and Henderson (1984) demonstrated that plasma osmolality and plasma concentrations of urea, sodium, chloride and other electrolytes were all decreased in environments of reduced salinity. The changes in plasma urea concentration were mediated by decreased blood production and increased

metabolic clearance, the latter effect being accomplished mainly by increased renal excretion. Similar decreases in urea biosynthesis have also been shown in both the little skate, Raja erinacea, and the lipshark, Hemiscyllium plagiosum upon adaptation to 50% seawater (Goldstein and Forster, 1971a; Wong and Chan, 1977).

Interestingly, Hazon and Henderson (1984) also demonstrated increased plasma osmolality and increased urea and electrolyte concentrations in Scyliorhinus canicula adapted to 140% seawater, the changes in plasma urea concentration being achieved by decreased metabolic clearance rate. These studies demonstrated that elasmobranchs osmoregulate by maintaining an hyperosmotic plasma and reinforced the experimental evidence which suggested that these changes were mediated predominantly by alterations in plasma urea and electrolyte concentrations.

2.2 Osmoregulatory Organs

2.2.1 Gill

The anatomy of elasmobranch gills has been studied by several authors (Kempton, 1969; Wright, 1973; Olson and Kent, 1980; DeVries and DeJaeger, 1984; Metcalfe and Butler, 1986). There are usually five pairs of gills although the frilled shark (Chlamydoselachus), the saw shark (Pliotrema) and the comb-toothed sharks (Hexachidae) may possess six or seven. Each gill arch or holobranch consists of a sheet of muscular and connective tissue (the interbranchial septum) which is supported by lateral rods of cartilage (the gill rays) which radiate out from the

cerato-branchialis (Marshall and Hurst, 1905). For most of their length, on both the anterior and posterior surfaces of the interbranchial septum, the gill filaments are attached and run radially outwards along the gill. The tip of each gill filament is usually free of the interbranchial septum. The major difference between elasmobranch and teleost gills is that in the latter the interbranchial septum is greatly reduced. On both the dorsal and ventral surfaces of each gill filament are arranged a row of (secondary) lamellae and these are the principal site of gas exchange.

The elasmobranch gill possesses all the characteristics associated with a respiratory surface and is highly impermeable to urea (Margaria, 1931; Hukuda, 1932), having the lowest reported urea permeability coefficient of any biological membrane (Boylan, 1967; Payan and Maetz, 1970; Payan, Goldstein and Forster, 1973). This is necessary because the large urea concentration gradient across elasmobranch gills, together with the large gill surface area, contributes to the gills being the major site of urea loss in these animals. Rates of trimethylamine oxide (TMAO) efflux at the gills are also very low and are comparable with those observed for urea (Goldstein and Palatt, 1974).

The extremely low urea permeability of elasmobranch gill branchial epithelium is not simply a reflection of a general impermeability of elasmobranch cell membranes to this molecule, as Fenstermacher, Sheldon, Ratner and Roomet

(1972) have shown in Squalus acanthias that urea rapidly equilibrates with a space equivalent to total body water volume. The "barrier" to urea efflux is not thought to be an (inward) active transport mechanism as it is unaffected by ouabain or chloromerodin, shows no detectable selectivity for urea over thiourea (Boylan and Lockwood, 1962), exhibits no temperature dependence over the physiological range and is not saturable (Boylan, Feldman and Antkowiak, 1963). It has been proposed that the unusually low urea permeability of elasmobranch gills is more likely to be the result of a "simple" physical property of the branchial epithelium (Boylan, 1967; Goldstein, 1982; Shuttleworth, 1988).

Elasmobranch gills are also the site of significant and consistent net ion uptake (Maetz and Lahlou, 1966; Horowicz and Burger, 1968; Bentley, Maetz and Payan, 1976) and do not therefore contribute to the overall elimination of excess salt gained from the environment and from the diet. Large concentration gradients exist for sodium and chloride ions between the environment and the body fluids and the absence of a substantial electric potential gradient (Bentley, Maetz and Payan, 1976) means that elasmobranchs are subject to continuous diffusional uptake of ions, primarily at the gills (Maetz and Lahlou, 1966; Burger and Tosteson, 1966; Horowicz and Burger, 1968; Payan and Maetz, 1973). Bentley et al. (1976) demonstrated that chloride ion influx was passive whereas sodium influx was not, and an active mechanism of sodium influx involving

Na^+/H^+ exchange has been suggested (Payan and Maetz, 1973; Evans, Kormanik and Krasny, 1979). Evans (1984) has suggested that a branchial Na^+/H^+ pump exists in all fish and probably plays a vital role in maintaining the acid-base balance of body fluids.

Carbonic anhydrase has been shown to be present in gill epithelia (Hodler, Heineman, Fishman and Smith, 1955) and the involvement of this enzyme in ion exchange has also been suggested (Payan and Maetz, 1973).

Efflux of both sodium and chloride ions also occurs at elasmobranch gills (Maetz and Lahlou, 1966; Horowicz and Burger, 1968; Bentley et al., 1976) but this is relatively small compared to the branchial influx of these ions. The presence of chloride cells has been reported in elasmobranch gill epithelia (Doyle and Gorecki,1961; Garcia-Romeu and Masoni, 1970; Wright, 1973) and these are structurally similar to those found in teleosts, although they are fewer in number and branchial Na-K-dependent ATPase activity (which is concentrated in chloride cells) was found to be ten to fifteen times less than that of a marine teleost (Jampol and Epstein, 1970). It is well established that chloride cells are the sites of active ion extrusion in the gills and other tissues of marine teleosts but a specific role for these cells in ion transport in elasmobranchs has yet to be determined (Laurent and Dunel, 1980). It can be concluded that if ion efflux across elasmobranch gills has an active component then this could be achieved by branchial chloride cells as in teleosts, but

such an efflux would be small and insufficient to render the gills a site of net ion excretion.

2.2.2 Gut

Considerable work has been done on the structure and innervation of the elasmobranch gut (for a review see Nilsson and Holmgren, 1988) but little is known of the functional role of the gut in osmoregulation. Marine elasmobranchs do not drink their environment under normal circumstances (Smith, 1931) and are believed to be intermittent feeders, so that epithelial transport mechanisms in the guts of these animals will be subject to brief periods of intense activity interspersed with longer periods of low activity or even inactivity. Seawater ingestion invariably accompanies food intake and it is probable that an absorptive mechanism for sodium chloride, perhaps involving Na-K-Cl cotransport similar to that observed in the flounder intestine (O'Grady, Palfrey and Field, 1987) may also operate in the gut of elasmobranchs. The absorption of water in fish gut is secondary to the active absorption of sodium and chloride.

Given that branchial water uptake and metabolic water production are sufficient to balance water loss and that the rectal gland excretes the ingested salt which accompanies food intake it is difficult to envisage a role for the elasmobranch gut other than the essential nutritional one. Clearly, only further investigation will determine whether the gut plays a functional role in osmoregulation in elasmobranchs.

2.2.3 Kidney

Elasmobranch kidneys are elongate paired structures situated in close proximity to the dorsal body wall. Renal arteries in Squalus acanthias arise as sixteen to eighteen ventral branches of the segmental arteries of the dorsal aorta and divide to form a network of vessels within the organ (Ghouse, Parsa, Boylan and Brennan, 1968). In the skate the pattern differs in that there is only one renal artery per kidney (Deetjen and Antkowiak, 1970). As in most non-mammalian vertebrates elasmobranchs possess a renal portal system. Portal veins are formed from the bifurcation of the large caudal vein, and on entry to the kidney divide and anastomose to form a matrix of small vessels. Portal blood mixes freely with blood from the glomerular vasa efferentia before leaving the kidney via the renal veins. There is also some evidence for a glomerular bypass vessel whereby blood may pass from afferent to efferent vessel, thus avoiding filtration (Green and Brown, 1986, cited in Henderson, O'Toole and Hazon, 1988).

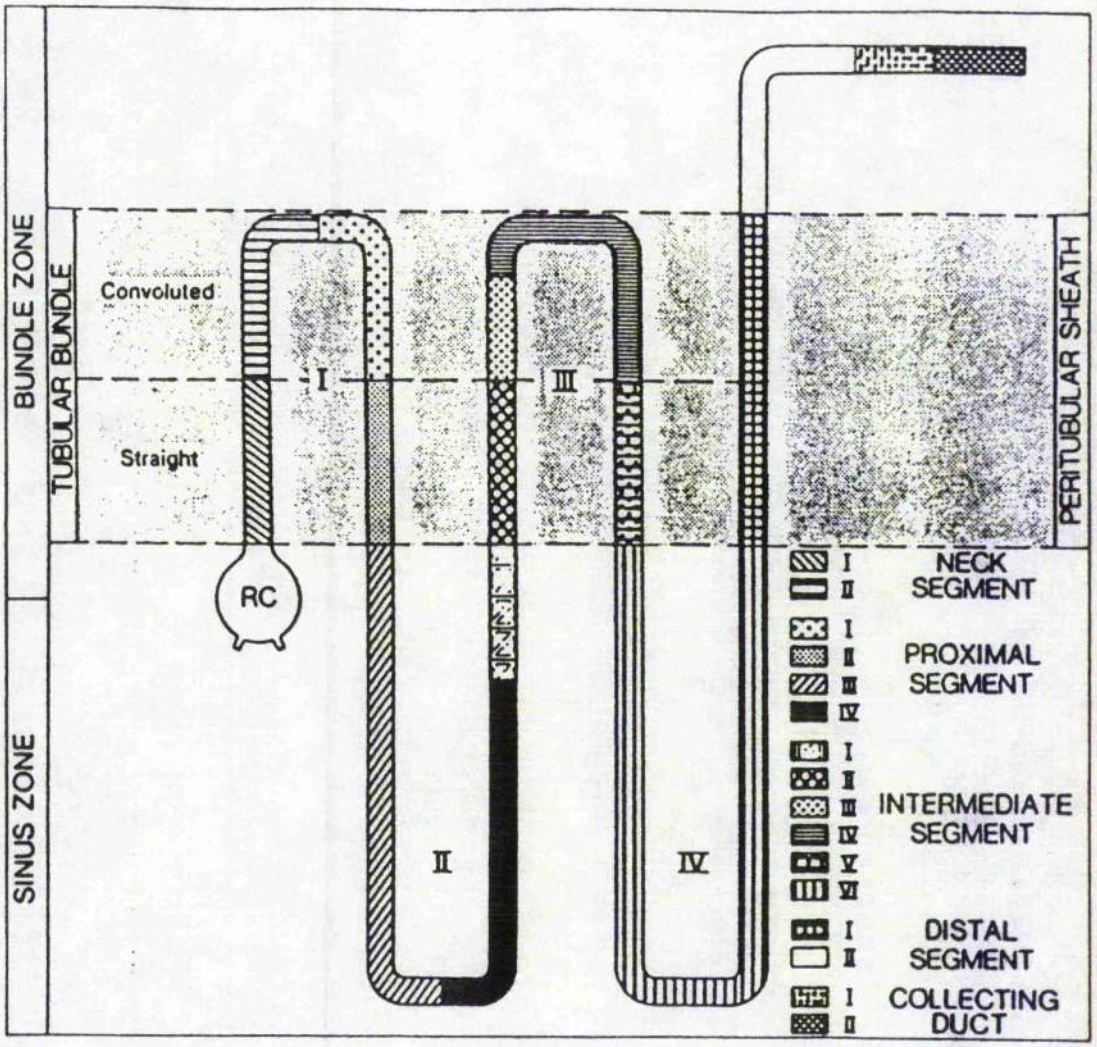
Elasmobranch nephrons are very long and highly complex in structure. This great length, the apparent regional heterogeneity in structure together with seasonal, sexual and species variations clearly hamper an easy understanding of the three-dimensional structure and basic function. However, a much clearer picture is now emerging, mainly through work on the little skate, Raja erinacea, in which renal tubular micropuncture is possible (Deetjen and

Antkowiak, 1970; Stolte et al., 1977; Lacy et al. 1985; Lacy and Reale, 1985 a,b).

The kidneys of Raja erinacea and Scyliorhinus canicula can be divided into two distinct zones, a dorsal "bundle" zone and a ventral "sinus" zone (Lacy and Reale, 1985a). Starting from the glomerulus the nephron is divisible into four discrete loops (loops I, II, III and IV), a distal tubule and a collecting duct (Figure 2.1). Among the characteristics that vary within these groups are overall tubular dimensions, type of epithelium, presence of a brush border, flagella and cilia, and mitochondrial density. The tubule can be thought of as folded back upon itself twice, two loops being contained within a peritubular sheath in the dorsal bundle zone of the kidney and two emerging into the ventral sinus zone. The loops enter and leave the sheath at the same point, running parallel for a distance before becoming highly convoluted. An elaborate countercurrent system is thus formed, within the peritubular sheath, from the ascending and descending limbs of loops I and III and the distal tubule, comprising five parallel segments surrounded by blood capillaries (Lacy and Reale, 1985 a,b). The glomerulus lies in the bundle zone, outside the peritubular sheath, while the remaining two loops (II and IV) lie in the sinus zone, entwined in the loops of adjacent nephrons. The peritubular sheath is made up of squamous epithelia linked by tight junctions and separates each bundle from the next, and apart from the point where the five parallel tube segments enter and the

Figure 2.1

Fig. 2.1 Schematic Drawing of the Skate Nephron
Schematic drawing of the course of two dorsal loops (I and III) and two ventral loops (II and IV), distal tubule segment and collecting duct of the skate nephron. Loops I and III and the (early) distal tubule form the tubular bundle (counter current system) and are wrapped by the peritubular sheath. RC, renal corpuscle. (From Lacy and Reale, 1985a).


BUNDLE ZONE
TUBULAR BUNDLE
Convoluted
Straight
SINUS ZONE
RC
I
II
III
IV
PERITUBULAR SHEATH
NECK SEGMENT
PROXIMAL SEGMENT
INTERMEDIATE SEGMENT
DISTAL SEGMENT
COLLECTING DUCT

point where the distal tubule leaves, the sheath is broken only by an afferent and efferent capillary (Lacy and Reale, 1985 a,b). The definitions used by Lacy and Reale (1985 a,b) present a considerable improvement on the sometimes inappropriate and unhelpful terminology used previously and may be utilised to relate elasmobranch renal structure to function.

The elasmobranch kidney reabsorbs 75-85% of filtered fluid and together with solute reabsorption this renders the urine hypo-osmotic to the plasma (Kempton, 1953; Schmidt-Nielsen and Rabinowitz, 1964). Marine elasmobranch GFR's are significantly higher than those found in seawater teleosts (Hickman and Trump, 1969) and changes in GFR appear to be mediated by changes in the number of filtering glomeruli rather than by change in single nephron filtration rates (Shannon, 1940; Kempton, 1953; Henderson, Brown, Oliver and Haywood, 1978). The sites and the neuroendocrine control of GFR are poorly understood and initial studies using pharmacological doses of adrenaline proved equivocal (Deetjen and Boylan, 1968; Forster, Goldstein and Rosen, 1972). More recently however, Brown and Green (1987), in a detailed study on Scyliorhinus canicula, demonstrated that adrenaline induced a clear glomerular diuresis, reflecting increased filtration rates in individual nephrons. It may be that changes in GFR can thus be mediated both by changes in filtration rate of individual nephrons and by variations in the numbers of nephrons active at a given time.

One important and impressive feature of the elasmobranch kidney is its ability to reabsorb urea (Marshall 1930; Smith, 1931 and 1936; Kempton, 1953). The sites and mechanism of this reabsorption are uncertain and there is conflicting and incomplete evidence for active and/or passive urea reabsorption.

Urea reabsorption is iso-osmotic and the fractional excretion of urea is only 0.5% under normal conditions, a figure remarkably similar to that found for the active reabsorption of glucose (Kempton, 1953). Elasmobranch nephrons will reabsorb 90-95% of filtered urea but only 35% of thiourea suggesting an urea-specific transport mechanism (Boylan, 1967). Urea reabsorption is inhibited by phloretin and chromate (Hays et al., 1977), both of which have been shown to inhibit active transport processes in mammals. Based on this, an active urea reabsorption mechanism was proposed (Smith, 1931; Kempton, 1953; Forster, 1970) and micropuncture studies have implicated loop II as a possible site of sodium-linked urea reabsorption (Stolte et al., 1977).

However, a passive transport mechanism has also been suggested. Kempton (1953) failed to demonstrate saturatable urea transport and similarly, Schmidt-Nielsen and Rabinowitz (1964) were unable to saturate the putative urea carrier mechanism using the urea analogues methylurea and acetamide. In addition probenicid, which blocks active urea secretion in the frog, does not affect urea reabsorption in the dogfish (Forster and Berglund, 1957).

Boylan (1967) proposed a passive countercurrent model for urea reabsorption which involves iso-osmotic, active reabsorption of sodium and water, together with tubular impermeability to urea in either or both loops II and III of the nephron. This would result in a low interstitial urea environment into which urea would passively diffuse from the urea-permeable distal segments, which are enveloped by the countercurrent foldings of loops I and III.

Ultrastructural studies have demonstrated that loop III (but not loop I) tubular cells have the characteristics of cells known to actively transport sodium in other tissues (Lacy, Schmidt-Nielsen, Galaske and Stolte, 1975; Endo, 1984). Further evidence to support this has recently been provided (Friedman and Hebert, 1990; Hebert and Friedman, 1990). These authors have demonstrated that the intermediate IV segment (in loop III) in the dorsal bundle zone of the peritubular sheath is a "diluting" segment in which active sodium chloride transport occurs via a Na-K-Cl cotransport mechanism with a stoichiometry of $1Na^+:2Cl^-:1K^+$. This segment exhibits some of the characteristics associated with a renal diluting segment in mammals and amphibians i.e. active sodium chloride absorption associated with a lumen-positive transepithelial voltage, reversible inhibition of sodium chloride absorption with loop diuretics such as furosemide, low transepithelial resistance ("leakiness") and negligible water permeability. In addition these authors have also demonstrated the

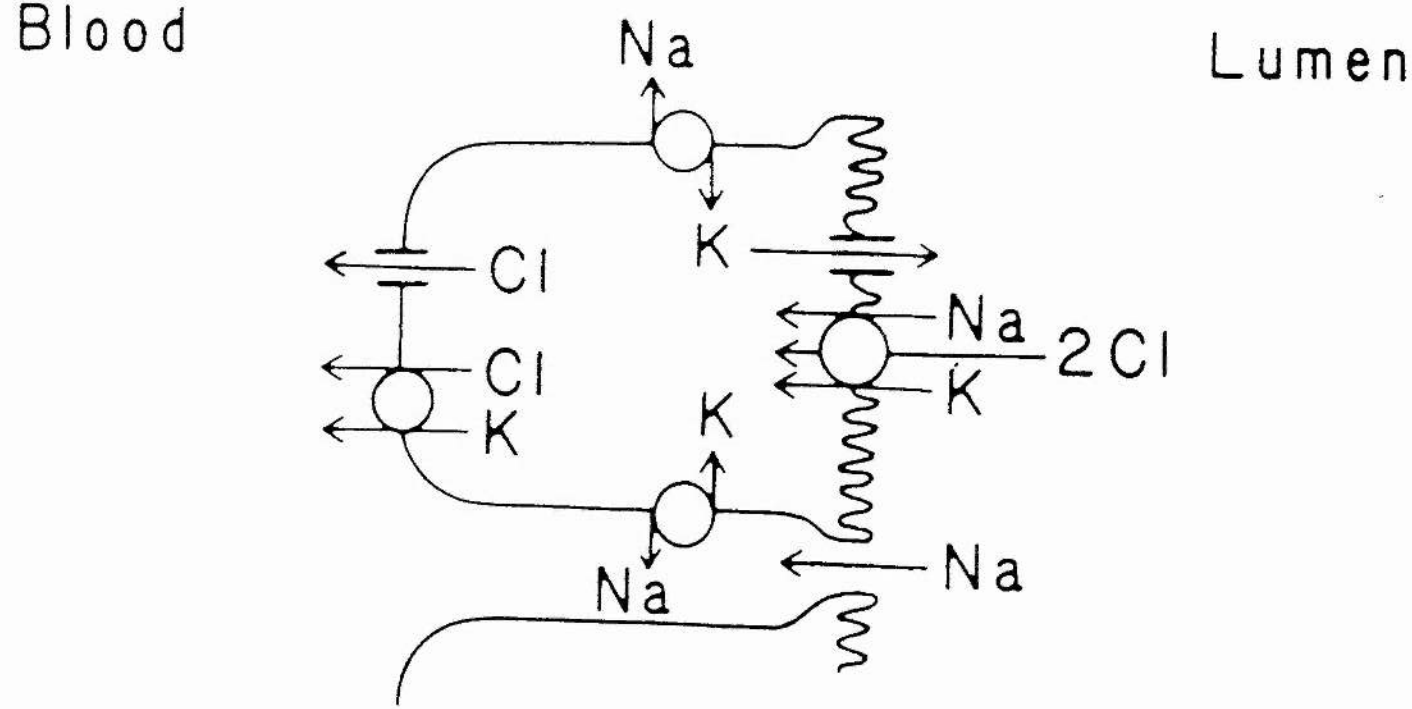
existence of an ouabain-sensitive Na^+K^+-dependent ATPase, probably located on the basolateral membrane, which presumably would function to keep intracellular sodium concentrations low thus maintaining the driving force for the Na-K-Cl cotransporter (Figure 2.2). On the basis of their work, Friedman and Hebert have proposed a "tentative" model for passive urea reabsorption in the elasmobranch kidney which extends the ideas put forward by Boylan (1972). Their model requires (1) a relatively proximal segment exhibiting high osmotic water permeability, (2) a relatively distal segment capable of high rates of active salt transport but effectively impermeable to water and urea, (3) a loop through the sinus zone having high hydraulic but low urea permeability, thereby permitting osmotic equilibration of the water leading to increased tubular concentration and (4) a relatively terminal nephron segment within the bundle zone exhibiting high urea but low osmotic water permeability, thereby permitting passive diffusion of urea from tubular fluid to interstitium within the bundle zone. The authors acknowledge however, that more evidence for some of the permeabilities proposed will have to be determined experimentally.

One key assumption of the Friedman and Hebert model is that the distal segment is both very permeable to urea and impermeable to water. This assertion has been supported by Thurau and Acquisto (1969), using tubular micropuncture, who suggested that the distal tubule was a site for urea reabsorption, but this was not confirmed by Stolte et al.

Figure 2.2

Fig. 2.2 Model of Sodium and Chloride Reabsorption at the Elasmobranch Kidney
Active chloride entry into the cell is coupled with the downhill entry of sodium via the electroneutral Na-K-Cl cotransporter. The resulting increase in intracellular chloride exceeds the elctrochemical equilibrium, creating a driving force for chloride exit through chloride channels; since the chemical gradient for potassium exit generally exceeds that for chloride entry a driving force for chloride exit through a K-Cl cotransport system also develops. The downhill gradient for sodium entry into the cell is maintained by the basolateral Na-K-ATPase which keeps intracellular sodium concentration low. Additional sodium is passively absorbed through cation-selective junctions between cells. (From O'Grady, Palfrey and Field, 1987).

Cl Absorption



(1977). These and other studies do agree however, that there is no urea reabsorption in the collecting ducts (Thurau and Acquisto, 1969; Deetjen, 1972; Stolte et al., 1977).

An additional and intriguing suggestion has been that the peritubular sheath may form a compartment of restricted diffusion or permeability, wherein the efficiency of the countercurrent exchange mechanism may be increased (Friedman and Hebert, 1990). Indeed, histological studies have shown that the sheath is comprised of flattened squamous cells joined by extensive tight junctions (Lacy and Reale, 1986) suggesting that it may be a semi-permeable membrane which would allow a microenvironment, separate from the surrounding tissue, to be developed (Lacy and Reale, 1986; Hentschel, Elger and Schmidt-Nielsen, 1986).

The precise nature of urea reabsorption in the elasmobranch kidney is still contentious but the recent evidence of Friedman and Hebert suggests the possibility that passive urea reabsorption following primary sodium chloride transport may be the functional mechanism. With the possible exception of Rana cancrivora (Gordon, Schmidt-Nielsen and Kelly, 1961) the mechanism of urea transport in elasmobranchs differs from that described for all other vertebrates.

The kidney is also of great importance in reabsorbing trimethylamine N-oxide (TMAO), a nitrogenous waste product and important osmotic constituent. To date little is known concerning TMAO reabsorption but analogies with renal handling of urea have emerged. As much as 90-95% of the

filtered oxide is reabsorbed (Norris and Benoit, 1945; Cohen, 1958) and tubular reabsorption is inhibited by structural analogues of TMAO such as trimethylamine and dimethylamine but not by methylamine. Cohen, Krupp, Chidsey III and Blitz (1959) have suggested that this indicates competition for a specific carrier.

Sodium is actively reabsorbed in elasmobranch nephrons (Boylan, 1967; Stolte et al., 1977) and renal micropuncture investigations have implicated loop II of the skate nephron to be site of both sodium and chloride reabsorption (Stolte et al., 1977). However, this section of the nephron does not have the cellular characteristics associated with active transport (Lacy, Schmidt-Nielsen, Galaske and Stolte, 1975; Endo, 1984). Histochemically, Na^+K^+-dependent ATPase activity has only been shown in the distal tubule and collecting duct (Endo, 1984). Tubular secretion has also been reported to occur in the elasmobranch kidney. The first example of tubular secretion was demonstrated by Smith (1939) in Squalus acanthias and was later confirmed in the smooth dogfish, Mustelus canis, by Kempton (1966). Isolated perfused loop II tubules from Squalus acanthias have been reported to actively secrete chloride ions (Beyenbach and Fromter, 1985) and this also appears to drive net fluid secretion (Sawyer et al., 1985 a,b). This may be interpreted as a renal mechanism supporting the rectal gland and may explain how elasmobranchs still osmoregulate and maintain normal plasma sodium and chloride concentrations following rectal

gland removal (Burger, 1965; Chan, Phillips and Chester-Jones, 1967).

The excretory patterns for chloride ions are influenced by plasma concentrations of other electrolytes such as magnesium, phosphate and calcium ions and also by urea. Anions such as phosphate and sulphate apparently reduce net urinary chloride secretion whilst magnesium promotes its excretion (Burger, 1967).

Net tubular secretion of divalent ions such as magnesium and phosphate and, to a lesser extent, calcium and sulphate has been demonstrated (Boylan, 1967; Stolte et al., 1977; Henderson et al.,1978) and micropuncture studies have suggested loop II as the site of this excretion (Stolte et al., 1977). It has been astutely pointed out that the ultrastructural studies used as evidence for active tubular reabsorption in loop II (Lacy et al., 1975; Endo, 1984) could just as easily be applied in support of active tubular secretion (Henderson, O'Toole and Hazon, 1988).

2.2.4 Rectal Gland

The elasmobranch rectal gland was first described by Hoskins (1917) and Crofts (1925) and its anatomy and histology have been extensively studied by a number of workers (Bernard and Hartmann, 1960; Doyle, 1962; Bulger, 1963; Chan and Phillips, 1967; Haywood, 1974; Kent and Olson, 1982). The rectal gland is a compound tubular gland consisting of many branched secretory tubules which drain into a duct opening into the intestine, posterior to the

spiral valve. Blood supply is usually via a single rectal gland artery derived from the post-mesenteric artery and blood is drained by the large dorsal intestinal vein (Kent and Olson, 1982). The cells that make up the secretory tubules contain numerous mitochondria and have greatly expanded basolateral membranes that typify cells involved in ion transport. In appearance they are similar to the salt secreting cells of avian and reptilian salt glands (Doyle, 1962; Bulger, 1963). The so-called tight junctions between secretory cells are quite shallow (Ernst, Hootman, Schreiber and Riddle, 1981; Forrest et al, 1982) and this is significant to the proposed mechanism of ion transport as sodium secretion is believed to follow a paracellular route.

In elasmobranchs the rectal gland functions in the elimination of excess salt by intermittently secreting a fluid that is essentially iso-osmotic with body fluids. This fluid which consists almost entirely of sodium and chloride ions at concentrations of approximately twice that found in the body fluids, whilst the urea content is only 10-20 nmol/l. This was first demonstrated by Burger and Hess (1960) who also showed that the gland produces this fluid in sufficient volume to effectively remove significant amounts of sodium and chloride from the plasma.

Accurate and reliable measurement of rectal gland secretion has proved difficult <u>in vivo</u>, partly because of technical problems and partly because of stress effects. Evidence also indicates that secretory rates in individuals, even under control conditions, vary quite markedly and rate

determinations have to be made over several hours (Burger and Hess, 1960; Burger, 1962; Holt and Idler, 1975).

Burger (1962, 1965) showed that rectal gland secretion in Squalus acanthias was stimulated by salt loading. Surgical removal of the rectal gland did not result in changes in plasma ion concentrations nor in urinary chloride ion concentration, but marked renal diuresis did occur and the overall urinary chloride loss was increased by 3-5 times (Burger, 1965). There was also some indication of decreased branchial ion influx and this was tentatively thought to be a result of some undefined change in branchial permeability. Following salt loading, restoration of normal plasma ion concentrations was delayed in glandless fish and Burger observed that without a rectal gland, sodium chloride is lost through increased urinary output and not by concentration of the urine above plasma levels. This necessitated a compensatory increase in water uptake, presumably at the gills, and it was concluded that the kidneys cannot serve as a substitute rectal gland (Burger, 1965). Chan, Phillips and Chester-Jones (1967), working on Hemiscyllium plagiosum, found similar results and observed that muscle sodium content, as well as plasma sodium concentration, was increased in glandless fish following a sodium load. Ligation of the rectal gland and urinary papilla of the pyjama shark, Poroderma africanum, produced increases in both plasma sodium and chloride

concentrations which diminished with time and were reversed when urinary constriction was released (Haywood, 1975a,b).

In view of the difficulties in evaluating rectal gland function in vivo many workers turned to the use of in vitro techniques. The most commonly used has been the in vitro isolated perfusion, first demonstarted by Palmer (1966), but tissue slices, membrane vesicles and isolated perfused segments of secretory tubule have also provided considerable insight into the mechanisms of transport involved and their control.

Biochemically rectal glands are rich in $Na,^{+}K^{+}$-dependent ATPase, an enzyme normally involved in active salt secretion (Bonting, 1966; Jampol and Epstein, 1970) and this enzyme was later localised to the basolateral membrane (Goertemiller and Ellis, 1976; Evelhoff et al., 1979). Early perfused gland studies showed that chloride secretion was against the prevailing electrochemical gradient (Hayslett, Schön and Epstein, 1974; Siegel, Schön and Hayslett, 1976) and this was later confirmed by Silva et al. (1977), using the stimulated rectal gland preparation, who also showed that chloride secretion was dependent on the presence of sodium in the perfusion medium and was inhibited by ouabain and furosemide. Furthermore, intracellular chloride concentrations were 4-6 times higher than those predicted on the basis of the electrochemical equilibrium across the basolateral membrane, a fact subsequently confirmed by microelectrode studies (Duffey,

Silva and Frizzell, 1978; Welsh, Smith and Frizzell, 1983).

Based on these data a model was proposed that the uphill entry of chloride across the basolateral membrane into the cell was coupled to the simultaneous downhill entry of sodium via a common cotransporter, that is inhibited by loop diuretics such as furosemide. The downhill gradient for sodium entry into the cell is maintained by the ouabain-sensitive $Na,^{+}K^{+}$-dependent ATPase located on the basolateral membrane keeping the intracellular sodium activity low. The resultant intracellular accumulation of chloride, to an activity greater than that predicted by the electrochemical equilibrium, is the driving force for its exit across the apical membrane into the secretory lumen, with sodium following passively via a paracellular pathway (Silva et al., 1977). The development of this model owed much to related work on transport in the avian salt-secreting gland (Ernst and Mills, 1977) and the mammalian ileum (Nellans, Frizzell and Schultz, 1973) and gall bladder (Frizzell, Dugas and Schultz, 1975), and is essentially similar to that used to describe chloride transport across a variety of vertebrate epithelia (Frizzell, Field and Schultz, 1979).

The original model has been modified as a result of work by Hannaffin, Kinne-Saffran, Friedman and Kinne (1983) who demonstrated that the basolateral cotransport system involves potassium ions, as well as sodium and

chloride ions, in a $Na^+:2Cl^-:1K^+$ configuration similar to that described in the thick ascending limb of the mammalian loop of Henle (Greger and Schlatter, 1983; Hannaffin and Kinney, 1983). The current model describing salt secretion in the elasmobranch rectal gland is shown in Figure 2.3.

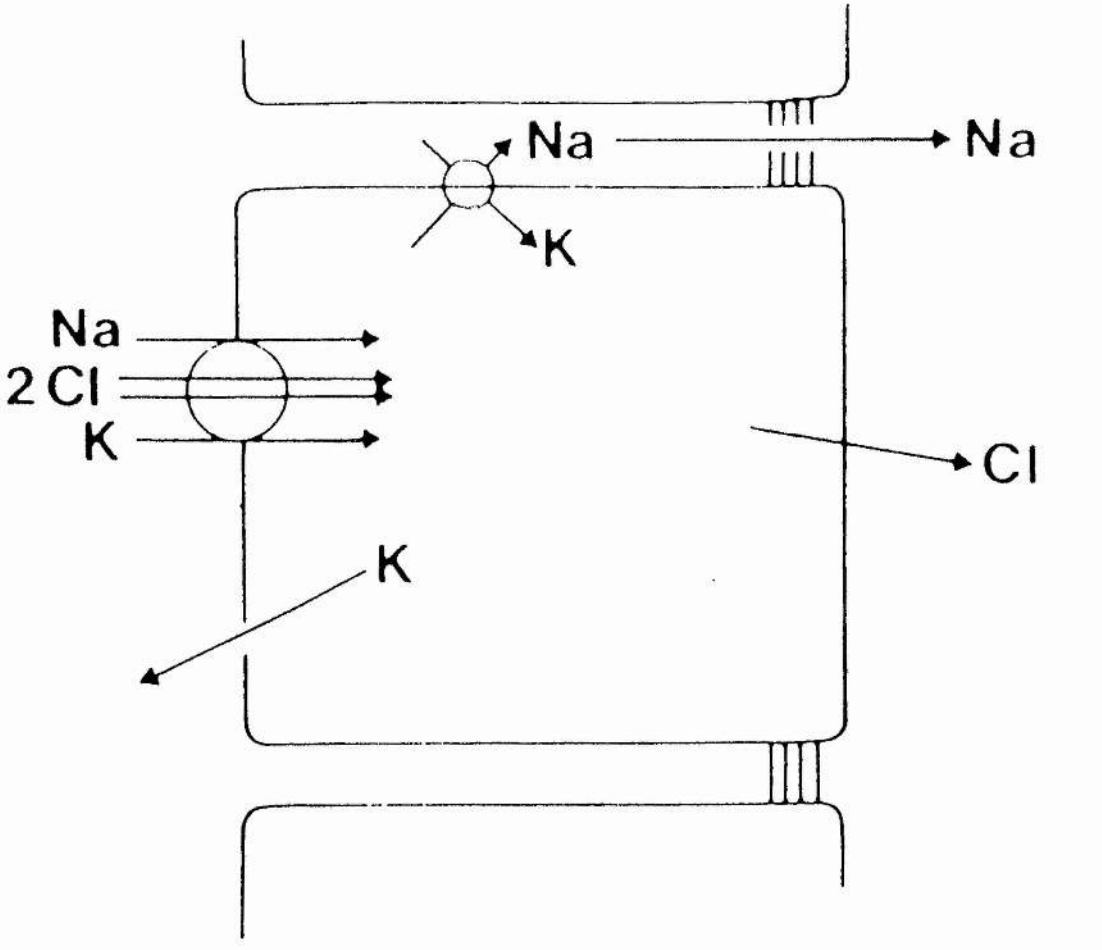
Rectal glands have also been shown to contain high carbonic anhydrase activity (Maren, 1962) but attempts to relate the function of this enzyme to secretory activity have proved equivocal. Recent evidence suggests that its major function is the elimination of metabolically produced carbon dioxide, rather than direct involvement in the secretory process (Swenson and Maren, 1984).

It was discovered in the perfused rectal gland that secretion was stimulated by membrane-permeable analogues of cAMP, by phosphodiesterase inhibitors such as theophylline which prevent cAMP breakdown (Stoff et al., 1977a) and later by forskolin, which directly stimulates adenylate cyclase to produce cAMP (Greger, Schlatter, Wang and Forrest, 1984). Subsequently the rectal gland has become a model system for the investigation of hormonal and second messenger systems in elasmobranchs.

Of the potential sites of action for cAMP it has been demonstrated by detailed microelectrode and patch clamp studies that the initial and specific action of cAMP is to increase the apical cell membrane chloride conductance by activating previously silent chloride channels (Greger, Sclatter, Wang and Forrest, 1984; Greger, Schlatter and Gögelein, 1985,1986, 1987). Recently Greger, Schlatter and

Figure 2.3

Fig. 2.3 Model of Salt Secretion by the Elasmobranch Rectal Gland
The uphill entry of chloride into the cell is coupled to the simultaneous downhill entry of sodium via the electroneutral Na-K-Cl cotransporter. The downhill gradient for sodium entry into the cell is maintained by the basolateral membrane Na-K-ATPase which keeps intracellular sodium activity low. The resultant intracellular accumulation of chloride to a concentration above the electrochemical equilibrium is the driving force for its exit through chloride channels in the apical membrane, with sodium following passively via a paracellular route.
(From Shuttleworth, 1988).


blood
lumen
Na
Na
K
Na
2 Cl
K
Cl
K

Gögelein (1987) and Gögelein, Sclatter and Greger (1987) demonstrated the existence of two chloride channels in apical cell membranes. The "large" channel requires stimulation to become active and is present in high frequency per area of luminal membrane when cells are stimulated. This channel can be blocked by agents that inhibit conductive chloride exit e.g. diphenylamine-2-carboxylate, and is thought to be responsible for hormone-dependent chloride exit from cells (Greger, Schlatter and Gögelein, 1987). The "small" channel may be active in both non-stimulated and stimulated states and may account for the low chloride conductance observed in the former state (Gögelein, Sclatter and Greger, 1987). The pronounced differences found between the properties of both the channels suggests that their concurrent presence in the same membrane may serve different physical needs within the cells.

Shuttleworth (1983a) has proposed that changes in intracellular calcium may be involved in the synchronisation of increased apical chloride conductance with increased Na-K-Cl cotransport since it has been observed that, although not mediated directly by calcium, the cAMP stimulation of secretory activity shows calcium dependency. Sodium influx is also increased in response to cAMP stimulation and this has been clearly shown to be a result of stimulation of the basolateral membrane Na-K-Cl cotransporter (Shuttleworth and Thompson, 1980b; Greger, Schlatter, Wang and Forrest, 1984).

Stimulation of secretion by cAMP also results in increased Na^+K^+-dependent ATPase activity (Shuttleworth and Thompson, 1978,1979,1980a; Silva, Stoff and Epstein, 1979) and although it has been claimed that this is partly a direct effect of cAMP on the pump, the bulk of evidence suggests that the increase in sodium pump activity is an indirect result of the cAMP-induced sodium infux via the Na-K-Cl cotransporter (Shuttleworth and Thompson, 1980b; Shuttleworth, 1982; Greger et al., 1984).

In summary, the effect of cAMP on the rectal gland is to increase secretion by directly altering apical chloride conductance synchronised, perhaps via calcium, with increased sodium influx via the Na-K-Cl cotransporter. This leads to increased basolateral Na^+K^+-dependent ATPase activity, maintaining low intracellular sodium which is the driving force for the cotransport system.

Bell and Sargent (1987) demonstrated the presence of high levels of protein kinase C activity in the rectal gland of Scyliorhinus canicula. The activity of this enzyme is controlled by the availability of DG, an intracellular second messenger produced during turnover of the inositol phospholipids. It has been shown previously that turnover of inositol phospholipids occurs when the rectal gland is in the resting, non-secreting state (Simpson and Sargent, 1985) and it may be that the postulated role of protein kinase C in the control of salt secretion (Bell and Sargent, 1987) is an inhibitory one.

The humoral control of rectal gland secretion in elasmobranchs has been the focus of considerable investigation over the past 15 years. The majority of work on the control of secretion by peptides has been carried out on a single species, Squalus acanthias, and the relevance of this work to elasmobranchs in general has been questioned (Shuttleworth, 1988)

Stoff et al. (1979) demonstrated that vasoactive intestinal polypeptide (VIP) stimulated salt secretion from the perfused rectal gland of Squalus acanthias, probably via a cAMP second messenger system, but initial attempts to show an analagous effect in vivo were inconclusive (Stoff et al., 1977b; Solomon et al., 1985a). More recently however, Silva et al.(1987) have shown that ANP release, in response to increased circulatory volume, stimulates rectal gland salt secretion by releasing VIP, thus demonstrating a physiological role for endogenous VIP. However, other workers using in vitro perifused rectal glands from Scyliorhinus canicula or Raja clavata were unable to stimulate secretion using heterologous VIP (Shuttleworth & Thorndyke 1984; Thorndyke & Shuttleworth, 1986) or using elasmobranch VIP (Dimaline & Thorndyke, 1986; Dimaline, Thorndyke and Young, 1986). The effect of homologous VIP has yet to be tested on rectal gland function in Squalus acanthias but is considered by Shuttleworth (1988) to be an unlikely candidate for the natural regulator of rectal gland secretory activity in elasmobranchs.

However, a peptide which is a potent stimulator of rectal gland secretion has been isolated from the intestine of Scyliorhinus canicula (Shuttleworth and Thorndyke, 1984; Thorndyke and Shuttleworth, 1986). This peptide, provisionally named rectin, is chemically unrelated to VIP and has been proposed as the endogenous regulator of rectal gland secretion in elasmobranchs. Given its putative important physiological role it is perhaps a little surprising that no subsequent work on this peptide has been published by the quoted authors or indeed by any other research group.

In Squalus acanthias, adenosine has been shown to stimulate rectal gland secretory activity at concentrations in excess of 10 μM (Erlij, Silva and Reinach, 1978; Forrest, Rieck and Murdaugh, 1980) and to inhibit secretory activity at concentrations less than 1 μM (Poeschla, Kelley, Boyer and Forrest, 1982). These responses may reflect effects on adenylate cyclase activity and result from adenosine binding to stimulatory (A2) and inhibitory (A1) purinergic receptor subtypes, respectively. Kelley, Nuland, Andreoni and Forrest (1985) suggested that adenosine may function as a feedback inhibitor during stimulation, operating via A1 adenosine receptors to prevent hypoxia and cell injury. This has recieved some support from the finding that secretory cells appear to possess only A1-type adenosine receptors (Shuttleworth, 1988) and these have recently been characterised pharmacologically (Kelly, Poeschla, Barron and Forrest,

1990). The same authors have also provided the first evidence for a high affinity A1 receptor that inhibits hormone- stimulated ion transport in a model epithelia (the rectal gland), and interestingly the major portion of the inhibition appears to be independent of the cAMP-messenger system. Of additional interest is the finding that the rectal gland vasculature of both Scyliorhinus and Squalus possesses vasodilatory adenosine receptors. These may also be part of the protective function of adenosine, their activation increasing rectal gland blood flow during stimulation and facilitating the removal of metabolites (Shuttleworth, 1983b).

Rectal gland secretion may also be under inhibitory control. In Squalus acanthias it has been demonstrated that somatostatin inhibits rectal gland secretion that has been stimulated by VIP, forskolin or cAMP, therefore indicating that at least part of its effect is exerted at a site distal to cAMP generation (Stoff et al., 1979; Silva, Stoff, Leone and Epstein, 1985). Most recently, Silva, Lear, Reichlin and Epstein (1990) have shown that heterologous bombesin inhibits rectal gland secretion by stimulating the release of somatostatin from rectal gland nerves. Histochemical studies have previously shown that VIP-,somatostatin- and bombesin-like immunoreactivities (as well as gastrin/cholecystokinin immunoreactivity) are present in rectal gland nerves, sometimes even within the same neuron (Holmgren and Nilsson, 1983). Peptidergic

control must be considered as one obvious mediator of rectal gland secretion *in vivo*.

Hormones acting on the adenylate cyclase-cAMP system are likely to modulate rectal gland secretion over a relatively short time period. The possibility of more prolonged regulation of secretory activity has been investigated by Holt and Idler (1975). Using *Raja ocellata* they demonstrated that following interrenalectomy, secretory rates of the rectal gland were reduced, a finding that could be reversed by injection of 1α-OH-B. Cytosolic receptor glycoproteins have been demonstrated in the rectal gland of the same species (Idler and Kane, 1980) and immunological studies have localised these receptors to parenchymal cells (Burton and Idler, 1986), which contain the Na-2Cl-K cotransporter and Na^+-K^+-dependent ATPase. A direct stimulatory role for the interrenal gland in the control of rectal gland function has been questioned by Hazon and Henderson (1984) who observed high levels of 1α-OH-B in intact *Scyliorhinus canicula* long term adapted to dilute seawater. In these fish plasma sodium and chloride concentrations had stabilised at a new lower level and rectal gland activity would be expected to be at a minimum. In addition, Chan, Phillips and Chester-Jones (1967), using *Hemiscyllium*, found that injections of cortisol and deoxycorticosterone decreased rectal gland secretion. Clearly the putative role of 1α-OH-B in the control of rectal gland secretion is unresolved and further work is obviously required.

It appears that in the rectal gland, as in other exocrine glands, an important relationship exists between secretory activity and the rate of blood flow to the gland. Under normal conditions in vivo the rectal gland may frequently be in a partially vasoconstricted state and stimulation of the gland to maximal rates of secretion is associated with simulataneous local vasodilation (Shuttleworth, 1983b; Solomon et al., 1984b; Shuttleworth and Thorndyke, 1986), as commonly seen in other exocrine glands. Initiation of secretion in vivo is likely to be triggered ultimately by change in blood or body fluid volume, since this is the principal factor involved in rectal gland secretion (Burger, 1985; Solomon et al., 1985b; Erlij and Rubio, 1986). Further evidence suggests that there are receptors in the region of the heart that responds to elevations in venous pressure by activating a pathway resulting in increased secretory activity in the gland (Erlij and Rubio, 1986). It has been suggested that this pathway may involve the release of ANP-related peptides from elasmobranch cardiac cells (Solomon, Solomon, Silva and Epstein, 1985; Silva et al., 1987). The effects of these atrial peptides are not directly on the secretory cells themselves but appear to be on vascular smooth muscle, to increase gland blood flow, and on rectal gland nerves to release VIP (Solomon et al., 1985; Silva et al., 1987).

In summary, the elasmobranch rectal gland intermittently secretes large quantities of sodium and

chloride ions at approximately twice their plasma concentrations in a fluid that is iso-osmotic with the plasma. It appears to be under complex humoral and peptidergic control and responds to an initial trigger of changes in blood or body fluid volume. Removal of the gland is gradualllly compensated for by alterations in kidney function and gill permeability, although these changes cannot be considered as replacements for the rectal gland per se. The rectal gland may be most important in removing acute salt excesses after feeding. Its overall osmoregulatory importance is unclear at present and should not be understated.

2.3 Urea

All marine and euryhaline elasmobranchs are ureotelic and urea is the major nitrogenous waste product of both extracellular and intracellular fluids. In both cases it accounts for approximately 35% of the total osmolality, and is regulated in response to varying environmental osmolality. The relative impermeability of the gill membranes and 90-95% renal urea reabsorption (Boylan, 1967) help to maintain high concentrations of urea in the body fluids. Urea and sodium are reabsorbed in a fixed ratio of 1.6 mol urea : 1.0 mol sodium (Schmidt-Nielsen, Truniger and Rabinowitz, 1972) and their reabsorptions have been linked. However, the differential effects of chromate on the renal reabsorption of both urea and sodium suggest that there may be some degree of independence in the reabsorptive mechanisms of both sodium and urea (Hays et al., 1977). Urea penetrates cell

membranes through aqueous pores (Goldstein and Forster, 1970) and is freely diffusable within the body tissues (Fenstermacher, Sheldon, Ratner and Roomet, 1972). Gill membranes are highly impermeable to urea and this is believed to be a physical barrier rather than an urea-retaining transport system (see section 2.2.1).

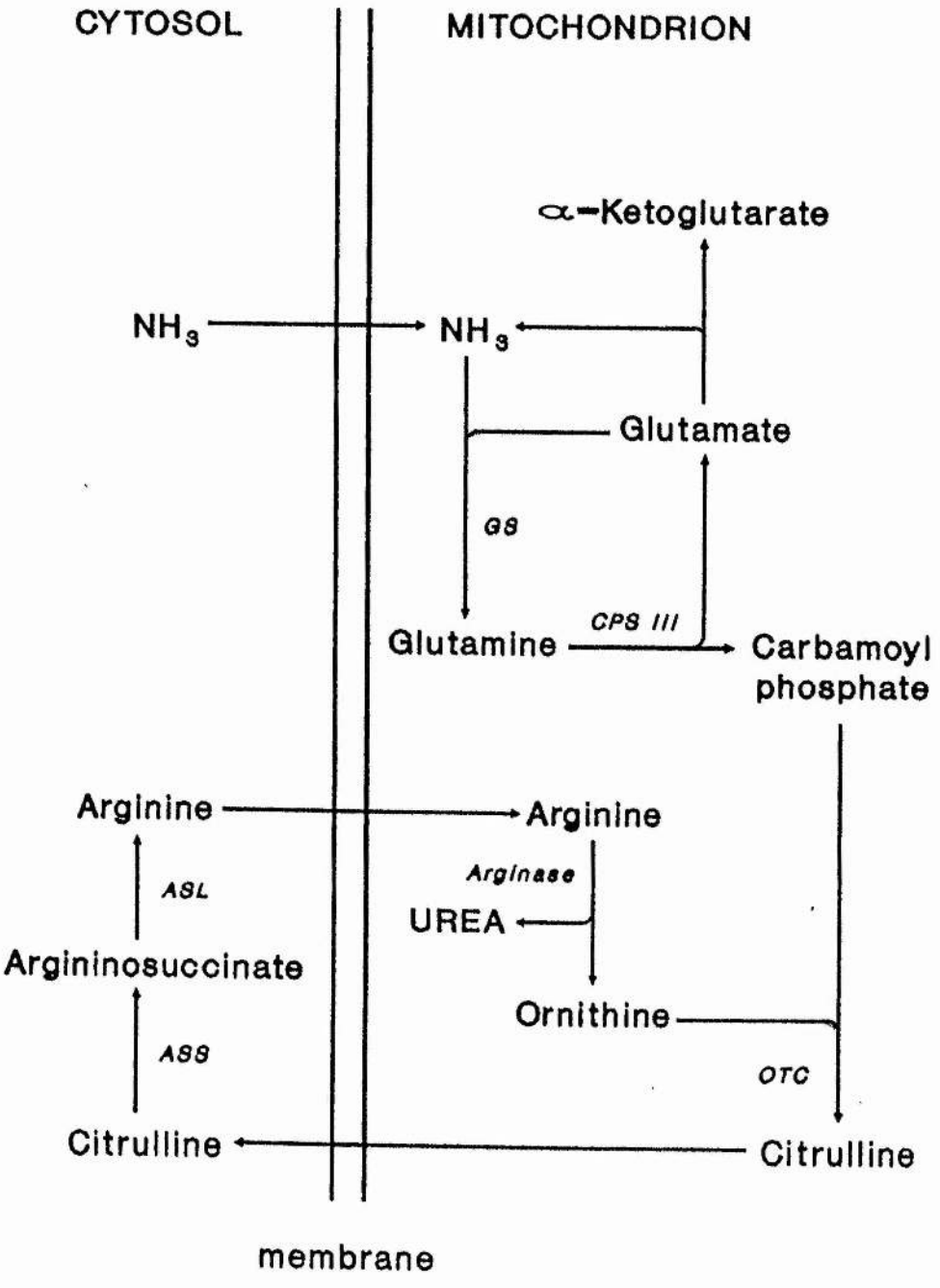
Urea loss occurs at the gills and to a lesser extent at the kidneys. Small amounts of urea are also lost intermittently during rectal gland secretion. In Squalus acanthias bacterial degradation of urea in the intestine has also been demonstrated but only contributes approximately 1% of total urea excretion (Lloyd and Goldstein, 1969). Urea loss in elasmobranchs is replaced by the biochemical synthesis of urea in the liver.

2.4 Urea Biosynthesis

There are three pathways for the synthesis of urea in elasmobranchs; (1) synthesis de novo from carbon dioxide and ammonia via the urea-ornithine cycle; (2) synthesis from the breakdown of purines; (3) synthesis from the breakdown of dietary arginine by arginase. Schooler, Goldstein, Hartman and Forster (1966), using in vivo and in vitro methods, determined that the hepatic ornithine cycle (Figure 2.4) was by far the most important route of urea synthesis in elasmobranchs although there was detectable urea synthesis via the purine pathway. The arginine pathway is probably of little quantitative importance in hepatic urea production because it is limited to the breakdown of dietary arginine. However, Campbell (1961)

Figure 2.4

Fig. 2.4 Pathway of Urea Synthesis in Elasmobranchs
Unique aspects of this pathway in elasmobranchs include carbamoyl phosphate formation from glutamine and the mitochondrial location of arginase.
(Modified from Anderson and Casey, 1984).


CYTOSOL
MITOCHONDRION
α-Ketoglutarate
NH3
NH3
Glutamate
GS
Glutamine
CPS III
Carbamoyl phosphate
Arginine
Arginine
ASL
Arginase
UREA
Argininosuccinate
Ornithine
ASS
OTC
Citrulline
Citrulline
membrane

demonstrated arginase activity not only in the liver but in the kidney and rectal gland of Mustelus canis and this pathway may have some significance in salvaging urea from arginine in extra-hepatic tissues.

All of the requisite enzymes for urea synthesis via the ornithine pathway have been demonstrated in elasmobranchs (Hunter and Dauphinee, 1924-5; Baldwin, 1960; Brown and Cohen, 1960; Campbell, 1961; Brown, 1964; Schooler, 1964) (Figure 2.4). One of the last enzymes to be demonstrated in this pathway was carbamoyl phosphate synthetase, shown first in the liver of the bonnethead shark, Sphyrna tiburo (Brown, 1964) and later in several other species of elasmobranchs (Watts and Watts, 1966). It has been shown subsequently that three types of carbamoyl phosphate synthetase (CPS) exist in nature, each distinguished by their nitrogen-donating substrate and cofactor requirements. CPS I is located in the liver mitochondria of ureogenic animals and requires ammonia as nitrogen-donating substrate and N-acetylglutamate (NAG) as a cofactor. The properties of this enzyme relate to its function in ammonia detoxification. CPS II is located in the cytosol of prokaryotes and simple eukaryotes. It requires glutamine as substrate but does not require NAG as cofactor. It is associated with pyrimidine synthesis. CPS III, like CPS II, requires glutamine as a nitrogen source but, like CPS I, requires NAG as cofactor. Anderson (1980) found very high levels of CPS III in livers of several elasmobranch species. Enzyme activity was found to be

tenfold greater with glutamine as substrate than with ammonia and was stimulated by the presence of NAG. Concurrent with these results was the discovery by Webb and Brown (1980) that hepatic and renal tissue of the same species of urea-retaining elasmobranchs also contained very high levels of glutamine synthetase activity. The correlation between glutamine synthetase and CPS III activities suggested a direct metabolic relationship between the production of glutamine and urea in elasmobranchs (Figure 2.4). This was supported indirectly by the findings that the freshwater stingray, Potamotrygon circularis, which does not retain high levels of urea, has very low levels of CPS III and low levels of liver glutamine synthetase (Anderson, 1980; Webb and Brown, 1980). Direct evidence for the relationship between glutamine synthetase and CPS III was produced by Anderson and Casey (1984) who demonstrated that; (1) citrulline (and hence urea) synthesis was abolished by the specific inhibition of the glutamine-dependent activity of CPS III with avicin, regardless of the nitrogen-donating substrate and; (2) citrulline synthesis was abolished by the specific inhibition of glutamine synthetase, with methionine sulfoximine, in the presence of ammonia and glutamate but not in the presence of glutamine.

Blood glutamine levels are not detectable in starved dogfish and are in very low levels in fed dogfish (Leech, Goldstein, Cha and Goldstein, 1979). There is, however, a significant net release of ammonia into the blood from tail

muscle in both freshly caught (and presumably fed) and starved (up to 22 days) dogfish (Leech, Goldstein, Cha and Goldstein, 1979) and ammonia must, therefore, be transported to the liver and kidney. Hepatic and renal glutamine synthetase can then convert ammonia to glutamine which can enter the hepatic urea-ornithine cycle via CPS III activity. Shankar and Anderson (1985) purified glutamine synthetase from Squalus acanthias and studied its structural and kinetic properties. In many respects this enzyme is similar to mammalian and avian glutamine synthetase but two unique properties were found, a very low apparent K_m for ammonia, and stimulation by halogen ions. The function of the halogen ion stimulation has not been explained but the low K_m for ammonia may be specifically related to the role of glutamine synthetase in providing glutamine for urea biosynthesis. The authors have suggested that the extra energy-requiring step of converting ammonia to glutamine and then using glutamine as a substrate for CPS III may provide a more efficient means of extracting ammonia from the blood, particularly at low concentrations. This would provide better regulation than if ammonia were assimilated directly to CP by an ammonia-dependent CPS. Anderson (1981) purified CPS III and studied its general and kinetic properties. He suggested that elasmobranch CPS III has unique properties which are related to its function in the synthesis of urea for osmoregulatory purposes rather than as a major route for disposal of ammonia from protein and amino acid catabolism.

Increases in urea concentration in the presence of TMAO results in significantly reduced glutamine synthetase and CPS III activities, when the concentrations of glutamate and NAG were not saturating. These effects appear to be due primarily to the inhibition of glutamine synthetase and it has been suggested that they might represent a homeostatic mechanism for maintaining physiological concentrations of urea (Anderson, 1981,1986; Shankar and Anderson, 1985) (Figure 2.4).

The third enzyme in the urea synthesis pathway, ornithine transcarbamylase (OTC), has been purified by Xiong and Anderson (1989) and its general and kinetic properties studied (Figure 2.4). It is similar to the OTC from mammalian ureotelic species but has an unusually low specific activity and is not sensitive to physiological concentrations of urea and TMAO. These results are in accordance with the view that OTC does not catalyse a rate-limiting step in the urea synthesis pathway and its properties do not appear to be unique to its role in the synthesis of urea for the purpose of osmoregulation (Xiong and Anderson, 1989).

The subcellular locations of the enzymes involved in urea synthesis have been determined (Casey and Anderson, 1982, 1985) (Figure 2.4). Glutamine synthetase, CPS III, OTC and arginase have all been demonstrated within the mitochondrial matrix and their location is consistent with their proposed sequential function (Casey and Anderson, 1985). A physiological explanation for the difference in

subcellular location of arginase between elasmobranchs (mitochondrial matrix) and mammals (cytosol) has not been established. Both ornithine and arginine are equally permeable to mitochondrial membranes suggesting that the mitochondrial location of arginine does not represent a unique mechanism for regulation of ornithine availability (and hence citrulline and urea synthesis). Also, citrulline synthesis proceeds at equal rates when using equivalent concentrations of either arginine or ornithine, even at low concentrations and the formation of arginine from ornithine is not rate-limiting for citrulline synthesis (Casey and Anderson, 1985). As in mammals the enzymes argininosuccinate synthetase and argininosuccinate lyase are located in the cytosol of elasmobranch hepatocytes (Casey and Anderson, 1982). The urea cycle enzymes are present in freshwater elasmobranchs but their activities are significantly lower than in those of marine elasmobranchs and body fluid urea content is also low (Goldstein and Forster, 1971b).

2.5 Trimethylamine Oxide

Trimethylamine oxide (TMAO) was first identified in dogfish muscle (Suwa, 1909) and later in the blood and urine (Hoppe-Seyler, 1930; Norris and Benoit, 1945; Cohen, Krupp and Chidsey III, 1958). Plasma TMAO concentrations are maintained within a narrow range, 60-80 mmol/l (Cohen, Krupp and Chidsey III, 1958). This is achieved by active reabsorption at the kidney, which retains approximately 90% of the filtered load, and by the marked impermeability of

the skin and gills to TMAO diffusion (Forster, Berglund and Renwick, 1958; Cohen et al., 1958; Goldstein and Palatt, 1974). In addition, there is a large muscle pool of TMAO and slow loss of TMAO from this pool contributes to the maintenance of constant plasma TMAO concentration (Goldstein and Forster, 1970). TMAO represents about 90% of the total methylamine concentration in elasmobranch plasma (Vyncke, 1970) and contributes about 60 mOsmol/kg to plasma osmolality.

Small losses of TMAO to the environment do occur but it remains unresolved whether this loss is replaced solely by dietary intake or by endogenous TMAO biosynthesis. Low rates of TMAO biosynthesis have been demonstrated *in vivo* and *in vitro* in the nurse shark, *Ginglymostoma cirratum*, and the lemon shark, *Negaprion breviostris* (Goldstein and Funkhouser, 1972; Goldstein and DeWitt-Harley, 1973), both of which inhabit warm waters. However, in similar studies on the spiny dogfish and little skate, Goldstein, Hartman and Forster (1967) were unable to show TMAO biosynthesis. Read (1968) demonstrated that TMAO biosynthesis occurred in the nutritionally closed system of the embryo of the big skate, *Raja binoculata*, showing, that in this species, TMAO biosynthetic ability is present, at least during embryogenesis. Goldstein and Palatt (1974) attempted to correlate the presence or apparent absence of TMAO biosynthetic ability in elasmobranch species with TMAO loss but were unable to show any relationship. Thus, it remains unresolved whether elasmobranchs have a common strategy for

maintaining body fluid TMAO in the face of environmental loss. It may be that evolutionary pressures, such as the availability of food (and hence the frequency of feeding), have determined whether a given species achieves its TMAO requirement through diet or endogenous biosynthesis. Clearly further investigation is required.

2.6 Ammonia

Ammonia is detectable in elasmobranch plasma (Leech, Goldstein, Cha and Goldstein, 1979) and its formation is believed to come from the deamination of amino acids, in a manner similar to that of other animals (Goldstein and Forster, 1970). Ammonia is excreted at the gills and in small amounts at the kidney (Cross et al., 1969). The major role for ammonia in elasmobranchs is its conversion to urea via glutamine and the urea-ornithine cycle (Anderson, 1980; Webb and Brown, 1980) (see section 2.4) but it may have an additional role in the regulation of acidosis (King and Goldstein, 1983b).

2.7 Counteraction of Urea Toxicity

The levels of urea normally observed in elasmobranchs would be considered "pathologically ureamic" in mammals and most other vertebrate groups. Normal elasmobranch concentrations of urea have strong perturbing effects on the structure and function of non-elasmobranch proteins and enzymes (Yancey and Somero, 1978; Yancey et al., 1982). The question is therefore, whether elasmobranch macromolecules have adapted to high urea concentrations to avoid such perturbations or whether there is some other

mechanism to counteract these effects. In some cases urea tolerance has been reported, as in elasmobranch haemoglobin (Bonaventura, Bonaventura and Sullivan, 1974; Martin et al., 1979), and some kinds of elasmobranch proteins appear to require urea for proper structure and function, e.g. the shark eye lens (Zigman et al., 1965) and M4 lactate dehydrogenase (Yancey and Somero, 1978). It is interesting that M4 lactate dehydrogenase from the freshwater elasmobranch species, Potamotrygon, which does not accumulate urea, does not require the presence of urea for optimal activity. However many elasmobranch enzymes lack urea adaptation (Malyusz and Thiemann, 1976; Simonarson and Watts, 1972; Cleworth cited in Yancey and Somero,1978) and the ability of these proteins to function was for many years not clearly understood.

Yancey and Somero (1979, 1980) demonstrated in vitro that methylamine compounds, especially TMAO, counteract the disruptive effects of urea in urea-sensitive elasmobranch and non-elasmobranch enzymes, and this is optimal at 2:1 urea:methylamine ratios. In vivo studies of intracellular urea and TMAO concentrations have shown that the measured ratio is near the 2:1 optimum. In addition Forster and Goldstein (1976) demonstrated that, while intracellular urea and TMAO concentrations decreased in Raja erinacea adapted to 50% seawater, the optimal urea:TMAO ratio was still maintained. Yancey et al., (1982) suggested that methylamine compounds have been selected (at least partially) as osmolytes because of their

stabilising effects on macromolecules in the presence of high concentrations of urea.

2.8 Amino Acids

Free amino acids form quantitatively important intracellular solute pools in numerous marine invertebrates (Gilles, 1975) and in several marine teleosts (Forster and Goldstein, 1979). Free amino acids are also important osmolytes in elasmobranchs contributing about 1% to the ECF osmolality and about 19% to the ICF osmolality (King and Goldstein, 1983a). Cells maintain themselves isosmotic with the ECF mainly through the regulation of amino acids. Only a few selected amino acids appear to be the major contributors to the high intracellular amino acid concentration in elasmobranchs and interestingly it is the concentrations of these amino acids, and not the entire body amino acid pool, that are modulated in response to environmental dilution (Boyd, Cha, Forster and Goldstein, 1977). The particular amino acids modulated vary depending on the tissue. In response to environmental dilution in the skate, sarcosine and ß-alanine are regulated in wing muscle and taurine and ß-alanine are regulated in red blood cells whereas taurine alone is regulated in the brain of the stingray. A possible reason for the intracellular accumulation and regulation of these specific amino acids as osmolytes is that they are relatively inert from a metabolic point of view (King and Goldstein, 1985a). They are not found in protein, so changes would not disrupt

protein biosynthesis, nor do they contribute to the major metabolic pathways of the cell.

Hypo-osmotic stress produces an increase in the release of amino acids from cells and using ^{14}C-taurine loading of skate erythrocytes Leite and Goldstein (1987) were able to demonstrate increased taurine efflux from cells in dilute medium. The increase in taurine efflux was associated with increases in the intracellular concentrations of DG and inositol-1-phosphate (McConnell and Goldstein, 1989), the products of phosphoinositide hydrolysis. DG is an activator of protein kinase C and it has been suggested that activation of this enzyme by DG during hypo-osmotic stress may result in the phosphorylation of a "taurine channel", altering its permeability to taurine and thus, ultimately contributing to the regulation of cell volume (McConnell and Goldstein, 1989; Goldstein, 1989).

2.9 Freshwater and Euryhaline Elasmobranchs

Traditionally elasmobranchs have been thought of as exclusively marine animals but true freshwater and euryhaline elasmobranchs do exist. Smith (1936) produced an extensive list of species that have been found in freshwater environments, although it has been found subsequently that many of the examples recorded represented rare or chance excursions into dilute media. Members of the ray family, Potamotrygonidae (*Potamotrygon*, *Elipesurus* and *Disceus*), are the only group of elasmobranchs that can genuinely be considered as predominately resident in

freshwater and are found particularly in the Amazon and Orinoco river systems, as far as 4,000 -4,500 km from the sea (Thorson, Cowan and Watson, 1967). These animals osmoregulate in a fundamentally different way from other marine or euryhaline elasmobranch species. Serum and body fluid levels resemble those of freshwater teleosts and plasma urea concentration is typically 1 mM whereas TMAO is undetectable (Thorson, 1967; Griffith, Pang, Srivastava and Pickford, 1973). The vast majority of nitrogenous waste is lost as ammonia and not urea (Forster and Goldstein, 1971a) which contrasts greatly with the urea:ammonia excretion ratio of 2:1 in the marine skate, Raja erinacea. Similarly, the excretion rate of injected ^{14}C-labelled urea by Potamotrygon was 45-50% per day compared with 1-3% by Squalus acanthias, indicating the inability of this freshwater species to reabsorb urea effectively at the kidney.

The Potamotrygonidae appear to osmoregulate mainly via electrolytes with the combined serum sodium and chloride concentrations nearly equivalent to serum osmolality. Several studies have shown that Potamotrygon can survive in salinities up to approximately half seawater, but urea accumulation plays an insignificant role, as urea biosynthetic ability and urea retention did not change (Thorson, 1970; Griffith, Pang, Srivastava and Pickford, 1973; Gerst and Thorson, 1977; Bittner and Lang, 1980). Potamotrygon possesses a vestigal rectal gland (Gerst and Thorson, 1977; Thorson, Wotton and Georgi, 1978),

supporting the notion of an ancestral marine origin for these truly freshwater species, but its function, if any, is not known.

A number of euryhaline elasmobranch species also exist and have been found on at least three continents. Carcharhinus leucas, found in Lake Nicaragua was initially considered landlocked but Thorson et al.(1971) demonstrated that specimens were capable of moving freely in both directions along the Rio San Juan, between the lake and the sea. Similar conclusions have been reached for several other species such as the sawfish, Pristis perotteti, from the same location (Thorson, 1967, 1982), P. microdon, C. melanopterus, Dasyatis warnak and Hypolophus sephen, all from the Perak River in Malaysia (Smith, 1931) and the Garoua stingray from the Niger and Benoue rivers of Nigera (Thorson and Watson, 1975). Euryhaline species resident in freshwater show relatively small reductions in plasma ion concentrations and still retain significant, albeit reduced, levels of urea in their body fluids (Urist, 1962; Thorson, 1967; Thorson, Cowan and Watson, 1973). Similar effects are seen in marine elasmobranchs tolerant of gradual dilution of the external medium (Goldstein and Forster, 1971a; de Vlaming and Sage, 1973). In most cases, the reduced urea levels seen in euryhaline species in dilute salinities result from a reduced rate of urea synthesis, and this can be reversed on exposure to normal seawater (Goldstein and Forster, 1971a; Wong and Chan, 1977). Euryhaline species possess the relatively low

permeability to ions and high water permeability typical of marine elasmobranchs (Carrier and Evans, 1973) and also have rectal glands of normal or reduced size which appear to be hypofunctional in freshwater (Oguri, 1964; Thorson, Wotton and Georgi, 1978).

2.10 Elasmobranch Endocrinology

The present studies are primarily concerned with the control of interrenal gland function and osmoregulation in elasmobranchs. This brief review will therefore concentrate on the glands and hormones directly involved in such control. Other elasmobranch hormones will be discussed more superficially.

2.10.1 The Hypothalamo-Hypophysial System

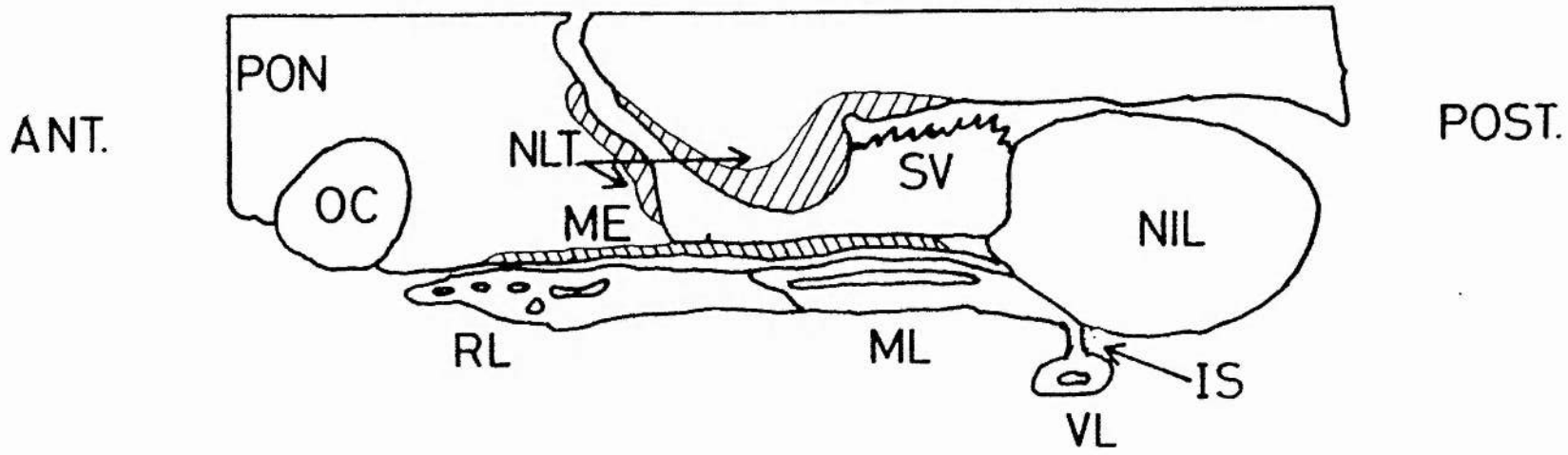
The elasmobranch pituitary appears to be unlike that of other vertebrates. The adenohypophysis is made up of a large pars intermedia and an elongated pars distalis. The pars intermedia is interdigitated with the neurohypophysis to form a neuro-intermediate lobe. The pars distalis consists of rostral, medial and ventral lobes, the latter being embedded in the floor of the cranium and connected to the rest of the adenohypophysis by a stalk (Figure 2.5).

In Scyliorhinus canicula the hypothalamo-hypophysial system was first demonstrated by Scharrer (1952) and Bergmann (1953). Nerve fibers originating in the preoptic nucleus of the hypothalamus terminate either on capillaries in the median eminence (Mellinger, 1960; Mellinger, Follenius and Porte, 1962; Meurling, 1967a) or innervate the neurointermediate lobe (Knowles, 1965; Meurling,

Figure 2.5

Fig. 2.5 Diagram of a Sagittal Section of a Generalised Elasmobranch Pituitary Complex

ANT : anterior
POST : posterior
RL : rostral lobe
ML : median lobe
VL : ventral lobe
NIL : neurointermediate lobe
SV : saccus vaculosus
OC : optic chiasma
IS : interhypophysial stalk
PON : preoptic nucleus
ME : median eminence
NLT : nucleus lateralis tuberis
(Modified from Hazon, 1982).


ANT.
PON
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VL

1967b). In addition, peptidergic fibers from the nucleus lateralis tuberis also supply the median eminence (Wilson and Dodd, 1973a; Holmes and Ball, 1974).

The elasmobranch median eminence has a rostral and a caudal region which supplies capillaries to the rostral and medial lobes of the pars distalis, respectively. The capillaries of the rostral median eminence are continuous with those of the rostral pars distalis whereas those of the caudal median eminence are true primary capillaries, connected to the secondary capillaries of the medial pars distalis via portal vessels. The caudal median eminence may also supply the neurointermediate lobe in Scyliorhinus canicula (Holmes and Ball, 1974; Ball, 1981).

The ventral lobe receives no portal blood supply and is not innervated and the only possible route for hypothalamic control of this lobe is via the systemic blood circulation.

The neurointermediate lobe receives two types of hypothalamic input: direct innervation by peptidergic and aminergic fibers and blood via the portal system. In Scyliorhinus canicula a neural lobe is recognisable but there is no neural lobe plexus and direct neurosecretion occurs in the pars intermedia (Scharrer, 1952). However, this arrangement varies depending on species (Meurling, 1967a).

All pituitary lobes receive arterial blood supply from the carotid sinus but the relative prevalence of portal and

arterial blood supply to the various pituitary lobes shows species variation (Meurling, 1967a).

2.10.2 The Adenohypophysis and its Secretions

2.10.2a Ventral Lobe

Thyroid Stimulating Hormone

Thyroid stimulating hormone (TSH) has been identified in the ventral lobe of the dogfish pituitary (Dodd, Ferguson, Dodd and Hunter, 1963) and the hormone purified and extracted (Sumpter, Follett, Jenkins and Dodd, 1978). Crude, homologous pituitary extracts have been shown histologically to stimulate dogfish thyroid gland function (Vivien, 1941; Olivereau, 1954) but, paradoxically, hypophysectomy had no histological effect on dogfish thyroid glands after 1 month (Waring, Landgrebe and Bruce, 1942) or even after 2 years (Dent and Dodd, 1961). However, pituitary ventral lobe cells reactive to thyroidectomy have been demonstrated (Alluchon-Gerard, 1978).

The control of TSH secretion is uncertain as there is no direct innervation or portal blood supply to the ventral lobe. Thyrotropin releasing hormone (TRH) activity has been found in the brain of Squalus acanthias (Crim, Dickoff and Gorbman, 1978; Jackson, 1979) and ventral lobe adenylate cyclase is activated by synthetic TRH in Scyliorhinus canicula (Deery and Jones, 1975).

The effect of the thyroid on basal metabolic rate has not been established as thyroidectomy of Scyliorhinus canicula produced no change in oxygen consumption (Matty,

1954). The thyroid gland has been linked with reproductive processes in several species (Ranzi and Zezza, 1936; Olivereau, 1949 a,b; Lewis and Dodd, 1974). Interestingly, an osmoregulatory role for the thyroid has been suggested by the findings that thyroidectomy of the Atlantic stingray, Dasyatis sabina, produced increases in both plasma osmolality and plasma urea concentration (de Vlaming and Sage, 1973; de Vlaming, Sage and Beitz, 1975). In vitro experiments also demonstrated that thyroxine decreased both renal Na^+-K^+-dependent ATPase and Mg^{2+}-ATPase activities with parallel reductions in the levels of cAMP and cGMP (Honn and Chavin, 1976).

Gonadotropin

Gonadotropic hormone (GTH) has been demonstrated in the ventral lobe of elasmobranch pituitaries (Dodd, Evennett and Goddard, 1960; Mellinger, 1964; Dobson and Dodd, 1977) and an annual cycle for GTH has also been demonstrated (Sumpter, 1976). The pituitary has been shown to be essential for reproductive processes in elasmobranchs (Dodd, 1972,1975) and it has been suggested that, similar to mammals, more than one gonadotropin may be involved (Dodd, 1975; Sumpter, Follett, Jenkins and Dodd, 1978).

2.10.2b Neurointermediate Lobe

Melanophore Stimulating Hormone

Melanophore stimulating hormone (MSH) is secreted by cells of the pars intermedia and originates from a macromolecular precursor protein, proopiocortin. Three forms of this hormone exist and MSH is known to influence

colour change in many non-mammalian groups (Batten and Ingleton, 1987).

The neurointermediate lobe has been demonstrated as the site of MSH secretion in elasmobranchs (Hogben, 1936; Waring, 1936; Landgrebe and Waring, 1941). The two common forms, α- and ß-MSH, have been extracted from the neurointermediate lobes of Squalus acanthias and Scyliorhinus canicula (Lowry and Chadwick, 1970; Love and Pickering, 1972,1974).

The neurointermediate lobe is under hypothalamic regulation and inhibitory aminergic fibers control MSH release (Meurling, Fromberg and Bjorklund, 1969; Meurling and Bjorklund, 1970) but a role for peptidergic innervation is uncertain. Cholinergic innervation has not been demonstrated (Wilson and Dodd, 1973b).

α-MSH immunoreactivity has been identified in the dorsal hypothalamic area of the brain of Scyliorhinus canicula and α-MSH may also act as a central neurotransmitter or neuromodulator within these animals (Vallarino et al., 1989b).

MSH controls the body colour response in elasmobranchs. Melatonin from the pineal gland is a potent paling agent and may also be important in body colour control (Wilson and Dodd, 1973b).

2.10.2c Median Lobe

Growth Hormone

Cytological (Della Corte and Chieffi, 1961; Mellinger, 1962a, 1966) and immunological (Lewis et al.,

1972) studies have demonstrated the presence of growth hormone (GH) in the median lobe of the elasmobranch pituitary. Immunological studies have indicated that shark GH is significantly different to GH from teleosts, with the exception of eel GH (Hayashida and Lewis, 1978). GH recently sequenced from the blue shark, Prionace glauca, shows considerable similarity to tetrapod GH (Yamaguchi et al., 1989).

It has been demonstrated that hypophysectomised fish do not grow (Vivien, 1941) but no definite physiological role for GH in elasmobranchs has yet been described.

ß-Pigment Dispersing Hormone

The octapeptide, ß-pigment dispersing hormone (ß-PDH) has also been demonstrated in the median lobe of the pituitary of Scyliorhinus canicula by immunohistochemical techniques (Vallarino, Feuilloley, Rao and Vaudry, 1990). This peptide, which produces rapid, reversible colour change in crustaceans, has not previously been demonstrated in vertebrates and its function in elasmobranchs is unknown.

2.10.2d Rostral Lobe

Prolactin

Prolactin, a hormone associated with osmoregulation in teleost fish, has been demonstrated cytologically (Della Corte and Chieffi,1961; Mellinger, 1962a), immunologically (Lewis et al., 1972; Grant and Banks, 1968) and by bioassay (Grant, 1961; Nicoll, Bern and Brown, 1966; Nicoll and Bern, 1968; Sage and Bern, 1970) in the rostral lobe of the

elasmobranch pituitary. Following rostral lobectomy plasma urea and sodium concentrations increased in Dasyatis sabina, an effect that was reversed by prolactin injections (de Vlaming and Sage, 1973; de Vlaming, Sage and Beitz, 1975). Prolactin injections also reversed the 50% decrease in branchial water permeability observed in Scyliorhinus canicula following hypophysectomy (Payan and Maetz, 1970), although ACTH had similar effects. Further studies on the physiological role of prolactin are required.

Adrenocorticotropic Hormone

Corticotropic activity has been demonstrated in the spiny dogfish, the skate and the stingray (de Roos and de Roos, 1967; Klesch and Sage, 1973, 1975) and the presence of adrenocorticotrophic hormone (ACTH) in the rostral lobe confirmed by immunofluorescence (Mellinger and Dubois, 1973). Elasmobranch ACTH has subsequently been extracted, purified and sequenced from the pituitary rostral lobe of Squalus acanthias (Lowry, Bennett and McMartin, 1974; Denning-Kendall, Sumpter and Lowry, 1982).

Squalus ACTH contains the same number of amino acids and is structurally very similar to human ACTH, differing by only two amino acid substitutions in the 1-19 amino acid region (Lowry and Scott, 1975), the region found to be all that is necessary for the full expression of steroidogenic activity (Maier, Barthe, Schenkel-Hullinger and Desalles, 1971).

However, the importance of ACTH in the control of elasmobranch corticosteroidogenesis has not been fully

clarified. Rostral lobe extracts from Squalus acanthias, Raja rhina and Dasyatis sabina were corticotrophic but the effect of hypophysectomy was variable (de Roos and de Roos, 1967; Klesch and Sage, 1973,1975). Interrenal atrophy followed hypophysectomy of Torpedo and Dasyatis sabina (Klesch and Sage, 1973) but was not evident in Scyliorhinus canicula, even after 1 year (Dodd, 1962). In addition, Hazon (1982) was still able to measure 1α-hydroxy-corticosterone in the plasma of Scyliorhinus canicula 21 days after hypophysectomy.

Experiments involving the administration of heterologous and homologous ACTH have produced a much clearer picture. In Scyliorhinus canicula both porcine and Squalus ACTH increased plasma levels of 1α-OH-B while dexamethasone produced a decrease (Hazon and Henderson, 1985). Using in vitro techniques Macchi and Rizzo (1962) were able to demonstrate increased steroid production from the interrenals of Raja erinacea following ACTH administration, although in a similar study on Raja rhina Bern, de Roos and Biglieri (1962) did not. In the nurse shark, Ginglymostoma cirratum, activity of the interrenal enzyme 3ß-hydroxysteroid dehydrogenase was increased following ACTH administration (Honn and Chavin, 1976). The interrenal gland is discussed further in section 2.10.9.

ACTH immunoreactivity has also been demonstrated in the caudal region of the basal hypothalamus as well as in the nucleus lateralis tuberis and the nucleus lobi lateralis of Scyliorhinus canicula. In the latter two

areas ACTH immunoreactivity is co-localised with another pro-opiocortin derived peptide, β-endorphin, and it has been suggested that both of these peptides may be involved in regulatory hypophysiotropic functions (Vallarino et al., 1989a).

2.10.3 Neurohypophysial Peptides

The elasmobranchs are particularly interesting in that they possess an unusual range of neurohypophysial peptides compared with other vertebrates. All species studied to date possess small amounts of arginine vasotocin (AVT) but skates and rays also produce an oxytocic principle, ^{4}Ser-^{8}Gln-oxytocin or glumitocin (Acher, Chauvet, Chauvet and Crepy, 1965; Acher, Chauvet and Chauvet, 1967; Chauvet, Chauvet, Beaupain and Acher, 1965; Sawyer, Manning, Heinicke and Perks, 1969). Sharks, on the other hand produce ^{8}Val-oxytocin (valitocin) and ^{4}Asn-oxytocin (aspartocin), although these have only been purified from Scyliorhinus canicula (Acher, Chauvet and Chauvet, 1972) (see Table 1.3).

Little is known of the physiological function of these peptides and the majority of studies have used either crude pituitary extracts or mammalian preparations. In Squalus acanthias rectal gland secretion was not altered following administration of arginine vasopressin, aspartocin, valitocin or AVT (Burger, 1962; Stoff et al., 1979) and total pituitary content of neurohypophysial peptide did not change on transfer to hypertonic seawater (Perks and Dodd, 1960). AVT, glumitocin, isotocin (the teleost oxytocin-like factor: see Table 1.3) and extracts of partially

purified neural peptide gave pressor responses in Scyliorhinus canicula (Payan and Maetz cited in Maetz and Lahlou, 1974). AVT and glumitocin were the most potent, up to 10 times more than isotocin or the neural peptide extracts. Unlike other fish, elasmobranchs appear to be able to alter their renal tubular water permeability and it has been suggested that a tubular-acting antidiuretic principle may be responsible (Henderson, Brown, Oliver and Haywood, 1978). Arginine vasopressin is the mammalian antidiuretic hormone and it is possible that the structurally related neurohypophysial peptides in elasmobranchs may have a similar function.

2.10.4 Neuropeptides

2.10.4a Galanin

Galanin, a 29 amino acid peptide, is found in the central and peripheral nervous systems of many vertebrates and has a range of central and peripheral actions. Galanin-like immunoreactivity has been demonstrated throughout the brain of Scyliorhinus canicula, including the preoptic nucleus, the nucleus lateralis tuberis and the floor of the hypothalamus adjacent to the median eminence (Vallarino, Feuilloley, Vandesande and Vaudry, 1990). It has been suggested that galanin may have neuromodulator and hypophyseotropic functions in elasmobranchs.

2.10.4b Neuropeptide Y

Neuropeptide Y (NPY) is a 36 amino acid peptide belonging to the pancreatic polypeptide family and was first characterised from porcine brain stem extracts by

Tatemoto (1982). It is extensively distributed throughout the central and peripheral nervous systems of mammals and may be involved in a number of neuroregulatory processes, both centrally and peripherally.

Extensive NPY-like immunoreactivity has been demonstrated throughout the brain of the dogfish, Scyliorhinus canicula, some of the highest densities being observed in the preoptic nucleus and nucleus lateralis tuberis, two areas of the hypothalamus involved in neurosecretion (Vallarino et al., 1988). Also, NPY nerve fibers originating from the preoptic neurosecretory complex in the hypothalamus have also been shown to innervate the pituitary neurointermediate lobe and further investigation will reveal whether NPY is involved in the (inhibitory) control of MSH secretion.

In addition to NPY neuropeptidergic control there is also some evidence of NPY neurohumoral control of the pituitary. NPY neurons have been observed in the nucleus lobi lateralis hypothalami, a visceral integration centre, and it has been suggested that NPY may be involved in the control of feeding behaviour in elasmobranchs (Vallarino et al., 1988).

NPY-like immunoreactivity has been demonstrated in the gut of several Raja species but nothing is known of its function in the fish gut (Bjenning and Holmgren, 1988).

Rich NPY-like innervation of the cardiovascular system of the little skate, Raja erinacea, has been demonstrated and suggests an important regulatory function for a NPY-

like peptide on vascular resistance (Bjenning et al., 1989). This suggestion has been given support recently by Hazon and Conlon (personal communication, 1990) who demonstrated a significant and sustained, dose-dependent pressor response to arterial injection of NPY in the lesser spotted dogfish, Scyliorhinus canicula. Bjenning, Driedzic and Holmgren (1989) failed to show NPY-like innervation of the cardiovascular system in Squalus acanthias but have suggested that this may reflect the relative dissimilarity of the putative Squalus NPY to the mammalian NPY against which the antiserum used was raised, rather than the complete absence of any NPY innervation of the cardiovascular system of this species. The presence of NPY-like nerve fibers in both the coronary vessels and myocardium has been demonstrated in Raja erinacea and Raja clavata and may indicate NPY involvement in the control of coronary blood flow and contraction of the heart (Bjenning, Driedzic and Holmgren (1989), similar to its role in mammals (Allen et al., 1983, 1986; Rioux, Bachel and Martel, 1986; Balasubramaniam et al., 1988).

2.10.4c Gut Peptides

Bombesin, gastrin/cholecystokinin (CCK), substance P, VIP and somatostatin innervation of the gut have been demonstrated in several elasmobranch species (Reinecke, Schluter, Yanihara and Forsmann, 1981; Holmgren and Nilsson, 1983a; El-Salhy, 1984; Holmgren, 1985; Bjenning and Holmgren, 1988; Bjenning, Jönsson and Holmgren, 1990). The primary effect of these peptides is on gut motility

(Falkmer et al., 1981; Lundin, Holmgren and Nilsson, 1984; Holmgren, 1985; Aldman, Holmgren, Jensen and Jönsson, 1986) although gastrin has also been shown to increase gastric secretion in the dogfish (Vigna, 1983) and bombesin can also increase gut blood flow via a direct effect on vascular smooth muscle (Bjenning et al., 1990). VIP, somatostatin and bombesin may also have an osmoregulatory role (Solomon et al., 1985a; Silva, Stoff, Leone and Epstein, 1985) being involved in the control of rectal gland secretion (see section 2.2.4).

2.10.5 Urotensin

Despite the fact that neurosecretory cells were first identified in the skate caudal spinal cord (Spiedel, 1919) the caudal neurosecretory system has been much less extensively studied in elasmobranchs than in teleosts. The teleost caudal neurosecretory system produces two distinct peptides: urotensin I is a large peptide similar in structure to mammalian CRF and to the frog skin peptide, sauvagine; urotensin II is a 12 amino acid peptide and is structurally similar to the biologically important central region of somatostatin-14. There is however, considerable species variation in the amino acid sequences of these peptides. In teleosts the urotensins may have an osmoregulatory role, controlling ion and water transport both directly and indirectly (Bern, 1985).

The presence of a caudal neurosecretory system in elasmobranchs has been established morphologically and its histochemical organisation has been described for several

species of elasmobranch e.g. the dogfishes, Squalus acanthias (Fridberg, 1962; Onstott and Elde, 1986), Squalus tarzame and Triakis scyllia (Owada, Yamada and Kobayashi, 1985) and the rays, Torpedo ocellata and Raja radiata (Fridberg, 1962). In these species nerve terminals are not concentrated into a compact urophysis but rather large caudal neurosecretory neurons project onto diffuse neurohaemal areas on the ventral surface of the posterior spinal cord (Fridberg and Bern, 1968).

Using antiserum to ovine CRF that showed full cross-reactivity to goby urotensin I, intense urotensin I immunostaining was observed in large polygonal cells in the caudal spinal cord region of Raja binoculata (Onstott and Elde, 1986). Dense urotensin I/CRF immunoreactivity has also been demonstrated on axons innervating blood vessels in the caudal spinal cord region of the banded dogfish, Triakis scyllia (Owada, Yamada and Kobayashi, 1985).

Antisera to goby urotensin II has identified caudal neurosecretory cells and axonal pathways containing urotensin II-like immunoreactivity in several species of dogfish and ray (Onstott and Elde, 1986; Owada, Yamada and Kobayashi, 1985). Purification and structural characterisation of an elasmobranch urotensin has not been reported but the immunohistochemical data provide firm evidence for the existence of peptides structurally related to teleost urotensin I and urotensin II in the elasmobranchs. It has been reported that the urophysis is

under serotonergic control in elasmobranchs (Onstott and Elde, 1986).

As a result of its morphology in elasmobranchs urophyseal ablation experiments are almost impossible and little work has been done on the physiology of elasmobranch urotensins.

2.10.6 Atrial Natriuretic Peptide

Atrial natriuretic peptide (ANP) is produced by mammalian atrial cardiocytes and has been associated with a variety of physiological actions, including vasodilation, marked natriuresis and diuresis and inhibition of both arginine vasopressin and aldosterone (for recent reviews see: Genest and Cantin, 1988; Brenner, Ballerman, Gunning and Zeidel, 1990).

Secretory granules similar to those found in mammalian atrial cardiocytes have been demonstrated in cardiac tissue (predominantly atrial) from Squalus acanthias (Solomon, Solomon, Silva and Epstein, 1985) and also in cardiac tissue from the electric ray, Narke japonica and the banded dogfish, Triakis scyllia, although the granule population was less dense in the latter two species (Uemura et al., 1990). In addition, using anti-α-human ANP antiserum, Uemura et al. (1990) were able to demonstrate ANP-like immunoreactivity in both atria and ventricles from the banded dogfish but not from the electric ray, although the latter result may be attributable to the low granule density. These authors also demonstrated that the amounts of immunoreactive ANP in ventricle and plasma extracts from

Narke japonica and Triakis scyllia were comparable with those of seawater teleosts studied, with the exception of Conger myriaster which had extremely high levels. The levels of immunoreactive ANP measured by Unemara et al. (1990) are however, at least tenfold less than those measured in Squalus acanthias and Dasyatis sabina by Evans, Chipouras and Payne (1989) who also used anti-α-human ANP antiserum.

Little is known of the physiological role of the putative ANP in elasmobranchs (see Table 1.11). Solomon, Solomon, Silva and Epstein (1985) demonstrated that both synthetic ANP and shark atrial extracts caused systemic hypotension and stimulated rectal gland secretion in vivo. In vitro the secretory response did not include a haemodynamic component. In addition, serum obtained after extracellular volume expansion contains a rectal gland stimulatory factor that is not present in serum before volume expansion. Later work by this research group has shown that ANP acts directly on peptidergic nerve terminals to stimulate VIP release, which in turn stimulates rectal gland secretion, and that this effect occurs concomittantly with decreased systemic blood pressure and increased rectal gland blood flow (Silva et al., 1987). These results are consistent with the hypothesis that a putative ANP is present in shark cardiocytes and is released during acute volume expansion. The ANP stimulates rectal gland chloride secretion, providing a negative feedback mechanism for the

regulation of extracellular volume (Solomon, Solomon, Silva and Epstein, 1985).

ANP has also been shown to produce vasodilation in vitro. Isolated ventral aortic rings from Squalus acanthias were vasodilated in response to physiological doses of $ANP_{(101-126)}$ and unlike mammalian preparations the shark aortic ring did not require preconstriction (Evans and Weingarten, 1989).

The kidney of Squalus acanthias was unresponsive to a dose of 2µg/kg $ANP_{(103-125)}$ when the shark was acclimated to normal seawater (Yokota and Benyajati, 1986) but injection of the same dose 24 hours after transfer to 70% seawater produced a significant stimulation of urinary chloride excretion (Solomon, Dubey, Silva and Epstein, 1988). Paradoxically, Benyajati and Yokota (1990) have recently demonstrated that injection or infusion of ANP produces decreases in GFR, urine flow rate and total osmolyte excretion in unrestrained Squalus acanthias in normal seawater. These authors have suggested that the volume status of the shark may modulate the effects of ANP on renal function.

There is increasing evidence, mainly from work in teleosts, that ANP may function primarily in salt homeostasis in fishes (Evans, 1990) but more work is clearly needed to clarify the renal effects of ANP and to elucidate the physiological role of ANP-like peptides in elasmobranchs (Benyajati and Yokota, 1990).

ANP-like immunoreactivity has recently been demonstrated throughout the brain of Scyliorhinus canicula, including the hypothalamus, preoptic nucleus, nucleus lateralis tuberis, and numerous fibers to the dorsal neurointermediate lobe (Vallarino et al., 1990b). These authors have suggested that there may be a neuromodulatory role for ANP as well as a hormonal one in elasmobranchs.

2.10.7 The Renin-Angiotensin System

The specific physiological role of the RAS varies among vertebrates but its overall functions are related to the regulation of blood pressure, body fluid volume and electrolyte balance (Taylor, 1977; Nishimura, 1978; Henderson, Oliver, McKeever and Hazon, 1981).

Cyclostome and elasmobranch fish are reported to be the only vertebrate group to lack a RAS (Nishimura et al., 1982). For elasmobranchs this view is based on a single pharmacological study and two histological studies. Nishimura et al. (1970) failed to demonstrate a pressor response in the rat bioassay following incubation of elasmobranch renal extracts and homologous plasma, and renal granular epitheliod cells have not been found in any elasmobranch species (Oguri, Ogawa and Sokabe, 1970; Sokabe and Ogawa, 1974).

However, responses to components of the RAS, typical in higher vertebrates, have also been demonstrated in elasmobranchs. Mammalian angiotensin I (AI) and angiotensin II (AII) were pressor when injected into Squalus acanthias and the response to AI was blocked by

captopril (Opdyke and Holcombe, 1976), analogous to the situation in many other vertebrate groups (Nishimura, Nakamura, Sumner and Khosla, 1982). The pressor response to AII was completely blocked by the α-adrenergic antagonist, phentolamine (Opdyke and Holcombe, 1976) and AII has been shown to release catecholamines in dogfish (Opdyke, Carroll, Keller and Taylor, 1981) so that the pressor response to AII in elasmobranchs, as in some other vertebrate groups, may be mediated by catecholamines. The significant vasopressor effect of AII in Squalus acanthias was not confirmed by Churchill, Malvin and Churchill (1985), who also failed to demonstrate any significant changes in GFR, urine flow and sodium excretion. Elasmobranch renal extracts incubated with rat renin substrate generated angiotensin-like pressor materials in the standard nephrectomised rat bioassay (Henderson, Oliver, McKeever and Hazon, 1981) and furthermore, elasmobranch renal extracts alone were pressor in the same bioassy suggesting renin-like activity. Recently, Masini, Henderson and Ghiani (1990) purified a fraction from renal extracts of Scyliorhinus canicula which had a molecular weight of 48,000 Daltons and displayed renin-like activity, acting on both synthetic and porcine angiotensinogen to produce AI. The fraction also showed similar electrophoretic mobility to mammalian renin. In addition, these authors also demonstrated angiotensin converting enzyme (ACE) activity in the spleen, gills, heart, liver, kidney, brain and rectal gland of Scyliorhinus canicula

which was inhibited by the mammalian ACE inhibitor, captopril.

The smooth muscle relaxant, papaverine, has been used in teleost fish as a means of stimulating the endogenous RAS (Balment and Carrick, 1985). Hypotension induced in Scyliorhinus canicula by papaverine was purportedly corrected by the activation of an endogenous RAS (Hazon, Balment, Perrott and O'Toole, 1989). A dipsogenic response accompanied vascular recovery and both can be blocked by captopril. However, captopril administration alone did not affect resting blood pressure or the low basal drinking rate suggesting that an angiotensin-like component is not involved in the maintenance of resting arterial blood pressure in Scyliorhinus canicula (Hazon et al., 1989).

Infusion of renal extracts and heterologous AII have also been shown to markedly increase plasma 1α-hydroxycorticosterone concentrations in dogfish (Hazon and Henderson, 1985).

Thus, it would appear that most of the requisite components of the RAS are present in elasmobranchs but clarification of the regulation and precise physiological roles of this system, as well as the identification and purification of the endogenous angiotensin-like principle(s) is obviously required.

2.10.8 Catecholamines

The elasmobranchs are distinct in having complete separation of adrenocortical tisssue and chromaffin tisssue, the latter being found in islets running the

length of the dorsal surface of the kidney and producing predominantly noradrenaline (West, 1955). Catecholamines may function in the control of the cardiovascular system in elasmobranchs as there is no direct sympathetic innervation of the heart (Young, 1933; Short, Butler and Taylor, 1977) and no motor innervation of the gill blood vessels (Metcalfe and Butler, 1984). Circulating catecholamine concentrations increase in response to hypoxia and stress (Butler, Taylor, Capra and Davison, 1978; Opdyke, Carroll and Keller, 1982) and may control gas exchange, either by altering gill blood flow (Davies and Rankin, 1973; Capra and Satchell, 1977; Evans and Claiborne, 1983; Metcalfe and Butler, 1984) or via a direct effect on the oxygen permeability of the gill (Isaia, 1984).

There have been few studies on the osmoregulatory effects of catecholamines in elasmobranchs and these have been restricted to the kidney. Early studies using largely pharmacological doses of catecholamines produced conflicting effects on elasmobranch kidney GFR (Deetjen and Boylan, 1968; Forster, Goldstein and Rosen, 1972) but more recently Brown and Green (1987) demonstrated a clear glomerular diuresis in response to adrenaline.

Catecholamines may also play a role in intermediary metabolism as their injection affects both lipid (Lipshaw, Patent and Fox, 1972) and carbohydrate metabolism (de Roos and de Roos, 1973), although carbohydrates may not be important as an energy source in elasmobranchs (Patent, 1975).

2.10.9 The Interrenal Gland

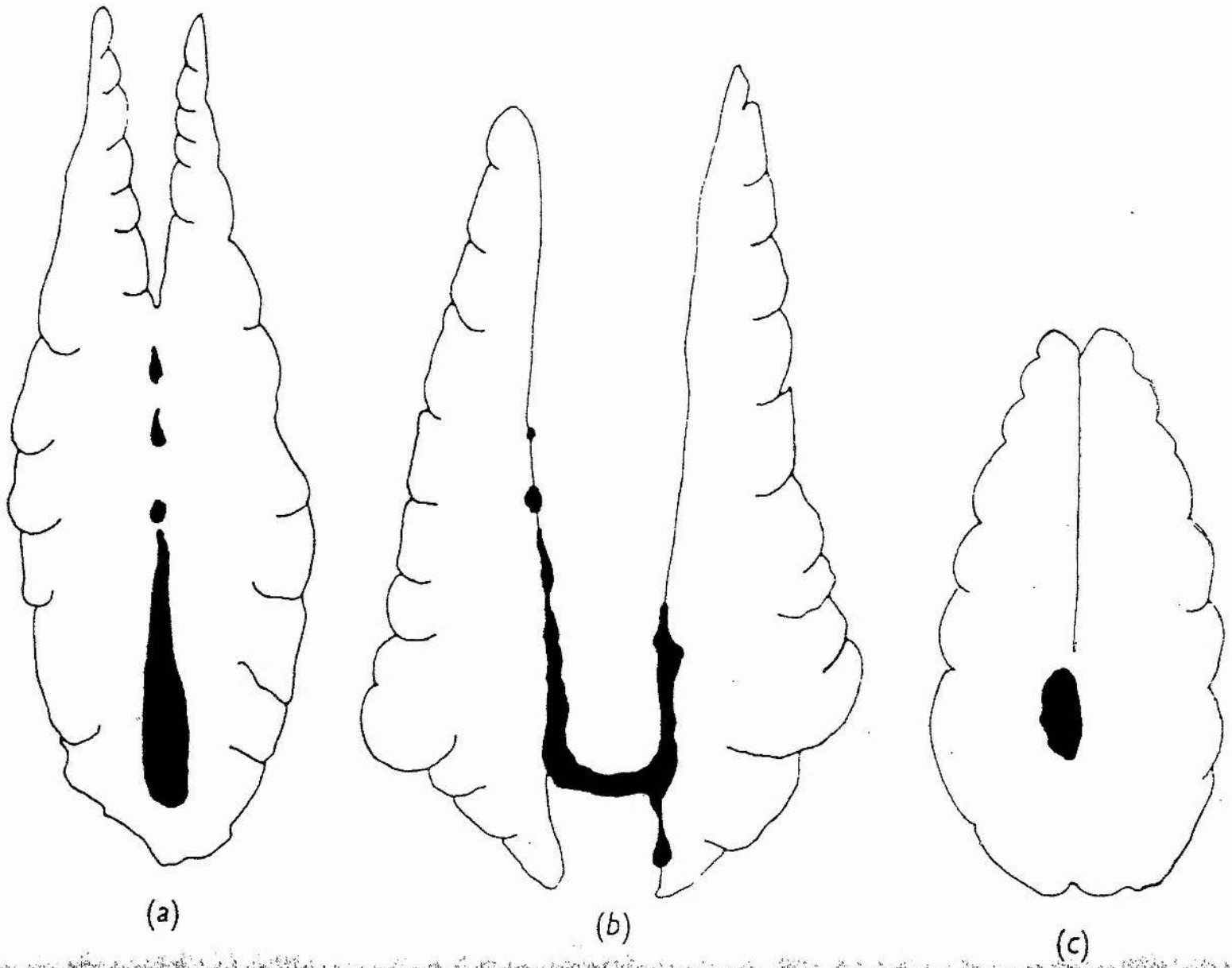
2.10.9a Structure

The elasmobranch adrenal homologue, the interrenal gland, normally lies dorsally between the 2 posterior lobes of the kidney. Morphologically it is comprised of pure cortical tissue. The chromaffin tissue is entirely separate, lying along the inner borders of the dorsal kidney surface in discrete islets (Chester-Jones, 1987). In teleosts and tetrapods there is a very close association between these two tissue types and only in cyclostomes is there a similar separation of chromaffin and adrenocortical tissue (Idler and Burton, 1976).

Figure 2.6 shows the three basic morphological types of interrenal gland that have been reported. The rod-shaped form, lying between the lobes of the posterior kidney, is found in sharks and holocephali whereas the horseshoe type is mainly found in rays: the oval body form, concentrated near the midline in the left kidney is mainly found in stingrays. Interrenal weights in mature male and female Scyliorhinus canicula have been reported to be in the ranges 20-101 mg/fish and 13-71 mg/fish, respectively (Roscoe, 1976). Roscoe (1976) has also reported that interrenal tissue in Raja species and in Scyliorhinus canicula consists of homogeneous cells grouped into cords or lobules. Zonation is largely absent in elasmobranch interrenals and has been reported in a single species Ginglymostoma cirratum (Taylor, Honn and Chavin, 1975).

Figure 2.6

Fig. 2.6 Variation in Form of the Elasmobranch Interrenal in Relation to the Kidney
(a) Rod-shaped type
(b) Horse-shoe-shaped type
(c) Concentrated type
(From Chester-Jones, 1957).


(a)
(b)
(c)

Interrenal activity varies little during the life cycle of elasmobranchs (Hartman et al., 1943; Oguri, 1960; Macchi and Rizzo, 1962) although early workers suggested that activity changes occurred at maturity (Ranzi, 1936; Fancello, 1937).

2.10.9b 1α-hydroxycorticosterone

Elasmobranch interrenal glands produce 1α-hydroxycorticosterone (1α-OH-B). 1α-OH-B was first identified in the plasma of elasmobranch fish by Idler and Truscott (1966a) and has subsequently been established as the major corticosteroid in all the elasmobranch species examined (Idler and Truscott, 1966b, 1967, 1969; Truscott and Idler, 1968; Simpson and Wright, 1970).

1α-OH-B is formed via the 17-deoxycorticosteroid sequence of reactions and results ultimately from the 1α-hydroxylation of corticosterone (Figure 1.7). Several authors have concluded that a 17-hydroxylating enzyme does not exist in elasmobranchs (Idler and Truscott, 1967; Sandor, Fazekas and Robinson, 1976) whereas the unique enzyme, 1α-hydroxylase, has been demonstrated in all 18 species of elasmobranch examined (Truscott and Idler, 1968; Idler and Truscott, 1972). 1α-hydroxylase is inactivated at 37°C and this has been suggested as a possible limiting factor for the presence of 1α-hydroxylase among the vertebrates (Idler and Truscott, 1966b; 1967).

As well as 1α-OH-B, small amounts of corticosterone, 11-deoxycorticosterone and 11-dehydroxycorticosterone have been reported as secretory products in vivo and in vitro

Simpson and Wright, 1970; Truscott and Idler, 1972). A radioimmunoassay for 1α-OH-B has been developed, using chemical conversion to 1-dehydrocorticosterone, and has permitted more detailed studies of this hormone to be carried out (Kime, 1975,1977; Hazon, 1982). Tissue binding studies using ^{3}H-1α-OH-B have demonstrated that the gills and the liver were the predominant binding sites (Moon and Idler, 1974) and cytosolic receptor glycoprotein receptors for 1α-OH-B have been demonstrated in the gills, liver, kidney and rectal gland of the same species (Idler and Kane, 1980). More recently an antibody raised against the 1α-OH-B binding protein has revealed that the gill receptor is probably located in chloride cells while the kidney receptor is associated with the section of the nephron possessing a brush border (Burton and Idler, 1986), corresponding to loop II in Lacy and Reale's (1985) definition. The rectal gland receptor was located in parenchymal cells which contain the salt secreting mechanism of this gland (see section 2.2.4).

The inaccessibility of the interrenal gland has been a consistant problem in the study of elasmobranch corticosteroid physiology. In those species where interrenalectomy is possible, surgery has produced inconclusive results with both experimental and sham-operated animals exhibiting very similar perturbations in plasma electrolyte composition before dying (Kisch, 1928; Dittus, 1939, 1941; Hartmann et al., 1944; Idler and Szeplaki, 1968). Upon recalculation of the Hartmann et

al.(1944) data Chester-Jones concluded that hypercalcaemia was the only true physiological response to elasmobranch interrenalectomy (Chester-Jones, 1957). de Vlaming, Sage and Beitz (1975) working on Dasyatis sabina reported reduced plasma sodium, chloride, calcium and urea concentrations following interrenalectomy. No sham operations (true controls) were carried out however, and the experimental animals were compared with intact ones so that the validity of this study is questionable.

A definitive range of functions for 1α-OH-B has not been clearly determined and investigations have aimed to establish whether interrenal steroids act on processes such as osmoregulation (mineralocorticoid activity) and intermediary metabolism (glucocorticoid activity). In heterologous bioassays, 1α-OH-B displayed mineralocorticoid activity, approximately 80% that of aldosterone in the isolated toad bladder (Grimm, O'Halloran and Idler, 1969) but had no gluconeogenic activity in adrenalectomised mice (Idler, Freeman and Truscott, 1967). The injection of exogenous steroids has also proved unenlightening. Cortisol and deoxycortisol produced no change in plasma sodium and potassium concentrations in the lip shark Hemiscyllium plagiosum although there appeared to be a reduction in the rectal gland secretory rate (Chan, Phillips and Chester-Jones, 1967). Interrenalectomised Raja ocellata showed reduced rates of rectal gland secretion and solute excretion which were rectified by injections of either 1α-OH-B or corticosterone (Holt and

Idler, 1975). The activity of Na-K-ATPase in the rectal gland was unaffected by either interrenalectomy or sham operation and the authors concluded that interrenal control of rectal gland function involved haemodynamic and/or permeability actions of the steroids.

No clear mineralocorticoid actions of 1α-OH-B have been demonstrated in elasmobranchs although an osmoregulatory role during osmotic stress has been suggested. Idler and Szeplaki (1968) observed impaired osmoregulatory function in interrenalectomised Raja radiata adapted to decreased seawater and Danowitch et al.(1975) suggested that the dramatic reduction in urinary potassium concentration 2 days after transfer to 70% seawater may be related to changes in corticosteroid levels. Hazon and Henderson (1984) showed that in dilute seawater Scyliorhinus canicula decreased plasma osmolality primarily by decreasing plasma urea concentration and that this was accompanied by increased plasma concentration, blood production rate and metabolic clearence rate of 1α-OH-B. Adaptation to 140% seawater produced increases in plasma urea and electrolyte concentrations but no change in plasma 1α-OH-B concentration or secretory dynamics. The authors suggested a role for 1α-OH-B in maintaining urea balance and postulated that the response was biphasic, consisting of a rapid phase renal diuresis and urea loss, possibly controlled by neurohypophysial peptides and/or catecholamines, followed by a slower phase involving decreased hepatic urea production, mediated by 1α-OH-B.

Interrenalectomy of Raja ocellata produced a transient hypercalcaemia (Idler and Szeplaki, 1968) and Hazon and Henderson (1985) observed hypocalcaemia and hypokalaemia following infusion of porcine ACTH, although a role for 1α-OH-B in calcium metabolism must remain conjectural at present.

Evidence for a glucocorticoid role is sparse, inconclusive and somewhat confused. Interrenalectomy of the skate significantly reduced hepatic liver glycogen (Hartmann et al., 1944) or had no effect (Idler, O'Halloran and Horne, 1969). The injection of exogenous steroids produced only a brief and transient hyperglycaemia (Wright et al., 1961) whereas in dogfish ten daily injections of either cortisol or corticosterone produced elevated blood glucose levels but no accompanying increase in liver glycogen levels (Patent, 1970). In Squalus acanthias, elevated blood glucose levels were reported after injections of mammalian ACTH although both sham and true hypophysectomy produced similar elevations (de Roos and de Roos, 1973, 1980).

Experimental evidence suggests that control of elasmobranch intermediary metabolism differs from that of other vertebrate groups. Injections of exogenous heterologous glucagon were without effect or produced only mild transient hyperglycaemia, even at high doses (Grant et al., 1969; Patent et al., 1970). Pancreatectomy had no consistent effect (Grimm, O'Halloran and Idler, 1969) and glucagon has not been isolated from the pancreas and is not thought to be produced by elasmobranchs. Insulin has been

isolated (MacLeod, 1922) and is hypoglycaemic (Leibson and Plisetskaya, 1968; Grant, Hendler and Banks, 1969; Patent, 1970) but as elasmobranchs tolerate both extremely high and extremely low circulating glucose levels it has been postulated that glucose may not be a required energy store (de Roos and de Roos, 1973; Patent, 1975). Patent (1970) suggested that because of the high liver lipid content, that studies of lipid and protein metabolism may be of more relevance and catecholamines may be of particular importance as they rapidly effect both lipid (Lipshaw et al., 1972) and carbohydrate metabolism (de Roos and de Roos, 1973).

Putative control of interrenal secretion by ACTH has been discussed in section 2.10.2d but little else is known of the control of interrenal steroidogenesis.

2.10.10 Second Messenger Systems

Little is known about the second messenger systems mediating cellullar functions in elasmobranchs. DG and protein kinase C may be involved in cell volume regulation in the skate (see section 2.8) but almost all of the published work has been on the control of rectal gland secretion (see section 2.2.4).

2.11 Objectives

The prime objectives of this study were to:

(1) Investigate the function of 1α-OH-B in elasmobranchs, and in particular its role in osmoregulation. In vivo osmotic adaptation studies were used to determine plasma

concentration and steroid dynamics of fish adapted to different dietary protein regimes.

(2) Identify, using an isolated in vitro perifusion technique, the putative factors and second messenger systems involved in the control of interrenal corticosteroidogenesis.

3. MATERIALS AND METHODS

3.1 Radioimmunoassay of 1α-hydroxycorticosterone

An antibody specific to 1α-OH-B has not yet been produced. The radioimmunoassay (RIA) employed in this study was based on the method of Kime (1977) and relies upon the conversion of 1α-OH-B to 1-dehydrocorticosterone and its subsequent RIA using an antibody raised to corticosterone.

3.1.1 Antibody Dilution

Duplicate assay tubes containing 100 µl of antibody at dilutions of 0 to 3000 were set up and then treated as normal assay samples, as described below. The percentage bound value at each dilution of antibody was plotted against the dilution factor to produce an antibody dilution curve.

3.1.2 Antibody Cross Reactivity

Duplicate 1ng samples of each potentially-cross reacting steroid were assayed and the percentage displacement compared to that of 1ng of 1-dehydro-corticosterone. The 1ng point was chosen for comparison because there is near-maximal displacement of ^{3}H-1-dehydrocorticosterone at this point on the standard curve. Percentage displacements for each steroid were calculated as follows:

$$\% \text{ Displacement} = \% B_0 - \% B_1$$

where $\% B_0$ = % binding at zero hormone concentration

$\% B_1$ = % binding of cross reacting steroid at 1ng

The cross reactivity (CR) was then obtained using:

$$CR = \frac{\%\ \text{Displacement of ng cross reacting steroid}}{\%\ \text{Displacement of ng 1-dehydrocorticosterone}}$$

Although 1α-OH-B is reported as the major corticosteroid in dogfish plasma it was still important to assess the extent of any cross reactivity between the antiserum and other steroids possibly present in the plasma. Thus, plasma and perifusion samples were also assayed for the presence of other corticosteroids and the cross reactivity of 1α-OH-B with these steroid antibodies calculated.

3.1.3 Assay Sensitivity

The sensitivity of an assay is defined as the least amount of measured material that can be distinguished from zero. A series of tubes containing only labelled steroid and antibody was assayed and the mean percentage binding plus or minus the standard deviation (S.D.) calculated. The amount of unlabelled material which produced a reduction of twice the standard deviation represented the assay sensitivity.

3.1.4 Intra- and Inter-Assay Variation

Extracted samples from a plasma pool were assayed as a matter of routine, with each set of unknowns and their mean and standard deviation calculated. The inter-assay coefficient of variation, a measure of assay reproducibility, was calculated using S.D./Mean x 100%. In a similar manner, repeat samples of the plasma pool were assayed within the course of a single RIA and their mean

and standard deviation calculated. The intra-assay coefficient of variation, a measure of variation within an assay, was then calculated using the above equation.

3.1.5 Standard Assay Procedure

100µl 1-dehydrocorticosterone standard were added to duplicate assay tubes and double diluted to form a standard curve of 0, 0.06, 0.12, 0.25, 0.5, 1.0, 2.0, and 4.0 ng. Extracted (see section 3.2), dehydrogenated (Kime, 1977) sample aliquots were pipetted in duplicate into plastic assay tubes and dried using a rotary evaporator (Rotavapor RE11, Buchi, Flawil, Switzerland). These samples were then reconstituted in 100µl BSA-saline (0.1% bovine serum albumin in 0.9% saline), ready for assay. 100µl BSA-saline, containing 10,000 d.p.m of ^{3}H-1-dehydro-corticosterone (specific activity = 22.8 Ci/mmol) were then added to each tube, the tubes vortexed and allowed to stand for 10 minutes at room temperature. Next, 100µl of antiserum at 1:1500 dilution were added to each tube and the mixture vortexed and allowed to stand for 30 minutes at room temperature. Tubes were then cooled on ice for 10 minutes prior to addition of 100µl of ice-cold charcoal-coated dextran (Separex tablets, Steranti Research, Ltd., St. Albans, Herts.). The tubes were vortexed and then left on ice for 20 minutes. The final assay volume was 400µl. Each sample was then centrifuged at 4^{o}C for 15 minutes at 2500g. 200µl of each sample supernatant were then added to 3ml of scintillation fluid (Emulsifier Scintillant 299^{TM}, Packard Instrument Co.Inc.,Illinois, U.S.A.) and

radioactivity counted for 5 minutes on a liquid scintillation counter.

Results are expressed as % Bound where,

$$\% \text{ Bound} = \frac{\text{d.p.m. bound in sample tube}}{\text{d.p.m. in total counts tube}} \times 100\%.$$

The 100% or total counts tube contained radioactive ligand and BSA-saline, made up to 400µl, but contained no antiserum. A standard curve of % Bound against 1-dehydrocorticosterone (ng) was then plotted and values for unknown samples read off the curve and corrected for recovery and volume.

3.2 Extraction of Steroids

Steroids were extracted from plasma and perifusion samples using SEP PAK C18 cartridges (Waters Associates, Millipore Corp., Northwich, Cheshire). Following the manufacturer's recommended procedure, the SEP PAKS were primed with 3ml methanol and washed with 5ml distilled water. Samples were then applied to the columns at a rate of 1-2 ml/min. The SEP PAKS were washed again with distilled water and the steroid then eluted at a constant rate with 4ml of methanol using an infusion/withdrawal pump (Harvard Apparatus, Massachusetts, U.S.A.). Using tritiated steroid as an indicator, the rate of methanol elution was varied in order to find the optimum for maximal recovery of steroid. All plasma samples were diluted with an equal volume of distilled water prior to application to the SEP PAKS. After use each SEP PAK was reconstituted using 5ml of 8M urea and then washed with 25ml of distilled

water. Treated in this way it was possible to reuse each SEP PAK four times without any appreciable decrease in steroid recovery.

In preparation for assay, sample extracts were acidified with 200µl of 2M sulphuric acid (in water-methanol, 1:1 v/v) and incubated for 3 hours at 60 ^{o}C to facilitate the quantitative conversion of 1α-OH-B to 1-dehydrocorticosterone (Kime, 1977).

3.3 Animals

Dogfish (Scyliorhinus canicula) of both sexes (600-1100g) were caught in the Firth of Clyde. They were maintained in aquaria at the Gatty Marine Laboratory, St.Andrews, in free-flowing, aerated seawater (osmolality, 960 mOsmol/kg; Na 399mM; K 8.5mM; Ca 12 mM;Mg 45 mM; Cl 368 mM) at 8-14 ^{o}C under natural photoperiod.

3.4 Reagents

Unless otherwise stated all of the reagents used were obtained from Sigma Chemical Co. (Poole, Dorset). Supplies of tritiated and non-tritiated 1α-hydroxycorticosterone and 1-dehydrocorticosterone and 1-dehydrocorticosterone antiserum were generously provided by Professor I.W. Henderson and Dr. D.E. Kime, University of Sheffield.

3.5 Development of Diets

The development of appropriate diets was performed in conjunction with Dr. C.B. Cowey at the Institute of Marine Biochemistry, Aberdeen. Pre-prepared ingredients were mixed in a Hobart commercial mixer and pellets of a suitable size prepared in an Alexander-Werk granulator.The

diets were then freeze-dried in a New Brunswick freeze drier. The first diet had a protein content of 11.35g per kg and the second diet a protein content of 5.68g per kg. Hereafter, they will be referred to as the high protein diet (HPD) and the low protein diet (LPD), respectively (Table 3.1).

3.5.1 Feeding Regimes

A successful feeding regime of 2.5g of diet per kg of fish every two days for thirty days was established, and fish readily ate both diets and maintained body weight.

3.6 Osmotic Adaptations

Upon adaptation to either a high or a low protein diet groups of fish were then acclimated, stepwise, to either 50% or 130% seawater over a period of fourteen days (Hazon and Henderson, 1984). The dietary feeding regimes were maintained during these osmotic acclimations. After the fourteen days, the dogfish were allowed a further five days before commencing metabolic clearance rate studies. 50% seawater was achieved by mixing seawater with increasingly greater amounts of freshwater in specially designed mixing tanks. 130% seawater was obtained by adding increasing quantities of sea salt to 30 gallon tanks in a re-circulating seawater system.

3.7 Surgical Procedures

Dogfish were anaesthetised in a 1:10,000 solution (w/v) of MS222 (tricaine methanosulphate, Sandoz Ltd.,Switzerland). During surgery fish were kept moist and cool but the gills were not irrigated.

Table 3.1

Table 3.1 Composition of Fish Diets

Table 3.1 Composition of Fish Diets

COMPONENT	LOW PROTEIN (g/kg)	HIGH PROTEIN (g/kg)
NORSEA FISH MEAL	368	737
FOSOL FISH OIL	218	22
EDIFAS (BINDER)	30	50
CELLULOSE	352.4	132.43
VITAMIN MIX	28	28
M1 MINERAL MIX	20	20
BHA	0.5	0.5
VIT A1, D3	0.07	0.07
SQUID MIX	10	10
TOTAL PROTEIN	250	500
TOTAL LIPID	257.5	100
DIGESTIBLE ENERGY		
PROTEIN	5.68	11.35
LIPID	9.2	3.58

3.7.1 Cannulation of Blood Vessels

A lateral incision immediately behind the left pectoral fin exposed the intestine and spleen. This gave access to the splenic vein and coeliac and anterior mesenteric arteries which were cannulated, where appropriate, with PE50 cannula (Portex tubing, Portland Plastics, Kent) containing heparinised dogfish Ringer solution (Appendix 2). The cannulae were held in place by a combination of purse-string sutures and miniclips (Autoclips, Clay Adams, Parsipanny, New Jersey, U.S.A.).

3.7.2 Collection of Blood Samples

During long-term experiments blood was drawn from arterial cannulae, microcentrifuged (MSE 25, MSE, Crawley, Sussex) and the plasma stored frozen at -20 °C until required. The blood cells were resuspended in an equal volume of dogfish Ringer solution, at the appropriate osmolality and pH, and reintroduced to the animals via the venous cannulae. When it was not possible to draw blood from cannula, samples were taken from the caudal sinus, approximately 2cm behind the cloaca, into a heparinised 2 ml syringe.

3.8 Measurement of Metabolic Clearance Rates and Blood Production Rates

Following cannulation, dietary and osmotically adapted fish were allowed 48 hours to recover before commencement of the isotopic infusion studies. The isotopic infusions were carried out as follows:

(i) 1α-hydroxycorticosterone: a priming dose of 5μCi of ^{3}H-1α-hydroxycorticosterone (specific activity = 23.9 Ci/mmol) was followed by a constant infusion of 0.5μCi/h at a rate of 6μl/min using a Harvard infusion/withdrawal pump (Harvard Apparatus). This led to equilibration within the plasma in 5-6 hours.

(ii) Urea: a priming dose of 10μCi of ^{14}C-urea (Amersham International PLC., Aylesbury, Bucks: specific activity = 55.2 Ci/mmol) was followed by a constant infusion of 1.0μCi/h at a rate of 6μl/min. This led to equilibration within the plasma in 5 hours.

Both isotopes were normally administered together, blood samples were taken periodically throughout the infusion, and the plasma counted for 5 minutes for tritium and carbon-14 using dual channel correction, on a liquid scintillation counter (2000CA Tri-Carb Scintillation Analyzer, Canberra-Packard, Pangbourne, Berks.). In addition 1α-OH-B and urea concentrations were also determined for the same plasma samples. In a similar study using longer time courses, Hazon and Henderson (1984) showed that no significant metabolism of the exogenous tracers occurred during the experiment.

The constant infusion of tracer molecules allows the determination of the metabolic clearance rate (MCR) of the endogenous material (Tait et al, 1962) and has previously been applied to teleosts (Owen and Idler, 1972; Henderson et al, 1974) and elasmobranchs (Idler and Truscott,1969; Hazon and Henderson, 1984 and 1985). The calculations, as defined by Tait et al (1962), are as follows:

At equilibrium, $MCR = I/X_c$ where

MCR = metabolic clearance rate in ml/h i.e. the volume of plasma irreversibly cleared of endogenous material per hour

I = constant rate of infusion in d.p.m./h

X_c = steady-state isotopic concentration in plasma in d.p.m./ml

Thus, knowing the rate of infusion and having measured plasma radioactivity, it was possible to calculate the MCR of the endogenous material. Blood production rate (BPR) was calculated from the observed MCR and the measured concentration of endogenous material, using the equation BPR = MCR x C, where C is the endogenous concentration, in ng/ml (for 1α-OH-B) or mmol/l (for urea), and BPR is in ng/h and mmol/h for 1α-OH-B and urea, respectively.

3.9 General Plasma Analyses

Plasma sodium and potassium concentrations were measured by flame emission spectrophotometry (Flame Photometer 450, Corning Ltd., Halstead, Essex). Plasma chloride concentration was measured using an automatic chloride titrator (Chloride Analyser 925, Corning Ltd., Halstead, Essex) and plasma osmolality by freezing point depression (Roebling Automatik, Camlab, Cambridge).

Urea

Plasma urea concentration was measured using a Sigma kit (Procedure No. 640) and is based on the following reactions: Urea is hydrolysed by urease to form ammonia and carbon dioxide. Ammonia then reacts with alkaline

hypochlorite solution and phenol in the presence of a catalyst, sodium nitroprusside, to form indophenol (blue). The concentration of ammonia is directly proportional to the absorbance of indophenol, measured spectrophotometrically (Cecil CE 599 Universal Automatic Scanning Spectrophotometer, Cecil Instruments, Cambridge). at 570 nm.

Cholesterol

Plasma cholesterol concentration was measured using a Sigma kit (Procedure No. 352) and is based on the following: cholesterol esters are hydrolysed by cholesterol esterase to form cholesterol. The cholesterol produced is then oxidised to cholest-4-en-3-one and hydrogen peroxide by cholesterol oxidase. Hydrogen peroxidase is then coupled with the chromogen, 4-aminoantipyrine and p-hydroxy-benzene sulphonate in the presence of peroxidase to yield a quinoneimine dye, which has an absorbance maximum of 500 nm. The intensity of colour produced is directly proportional to the total cholesterol concentration in the sample.

Protein

Protein concentrations were measured using the method of Bradford (1976). Briefly, 100 mg of Coomassie Brilliant Blue G250 dye were dissolved in 50 ml of 95% ethanol (James Burroughs Ltd., Witham, Essex). 100 ml of 85% (w/v) orthophosphoric acid (BDH Ltd.) were added and the volume made up to 1 litre using distilled water. 3 ml of a 1:2 dilution of this stock were then added to 100 µl samples

and BSA standards ranging from 0-100 µg. The blue colour formed after 10 minutes was stable for an hour and samples and standards were measured spectrophotometrically at 595nm. A standard curve of µg BSA against absorbance at 595nm was plotted and sample values for absorbance extrapolated to give protein content.

Lipid

Total plasma lipid content was determined using a modified Folch procedure (Ways and Hannahan, 1964; Christie, 1987) and all operations were carried out on ice or at 4°C. 10 ml of methanol were added to 1ml of plasma and the mixture vortexed thoroughly for 1 minute. 20 ml of chloroform were then added and the mixture again vortexed vigorously, in a fume hood, for 1 minute to precipitate proteins. The mixture was then centrifuged for 5 minutes at 80g and 4°C to pellet the precipitated proteins. The supernatant was retained and the pellet washed in 15ml of chloroform-methanol (2:1, v/v) and centrifuged as above. The two supernatants were combined and transferred to a clean, glass measuring cylinder. One quarter volume of 0.88% potassium chloride in water was added, mixed vigorously and the mixture allowed to settle. Two layers partitioned, an upper aqueous layer containing gangliosides and other non-lipid contaminants and a lower solvent layer containing pure lipid (Ways and Hanahan, 1964; Christie, 1987).. The aqueous layer was drawn off by aspiration, one quarter of the volume of the lower layer of methanol-saline solution (1:1, v/v) added and the washing procedure

repeated. The upper layer was again removed by aspiration and the solvent was then removed from the lower layer (containing the purified lipid) by evaporation on a rotary evaporator. The lipid was then stored in a small volume of chloroform at -20 °C until further analysis. Using this method a quantitative extraction of lipid is obtained (Christie, 1987). Aliquots of lipid extract were then dried onto accurately pre-weighed filter papers (Whatman No.3, Whatman International Ltd., Maidstone, England) and re-weighed accurately on an electronic microbalance (ME22, Metler Instruments A.G., Greifensee-Zurich, Switzerland).

3.10 In Vitro Experiments Using Dogfish Liver Tissue

A series of techniques were used in order to measure *in vitro* urea production rates and hepatic urea-ornithine cycle enzyme activities.

3.10.1 Urea Production Rates

Dogfish liver was dissected and then:

(1) Thinly sliced using fine dissection scissors.

(2) Homogenised in ice-cold Ringer solution using a glass, hand-held homogeniser.

(3) Prepared as isolated hepatocytes.

In the latter case the procedure used was a modification of that used by Mommsen and Moon (1987) and is summarised in Figure 3.1. The liver was left *in situ*, the hepatic vein cannulated and the liver perfused for 1 hour with modified dogfish Ringer solution, at a rate of 12ml/min, to remove red blood cells. The solution used was calcium-free, contained 0.4 mM EGTA, 5 mM Hepes and had

Figure 3.1

Fig. 3.1 Summary of Hepatocyte Isolation and Urea Assay Procedures
(From Mommsen and Moon, 1987).

Fig. 3.1 Isolation of Hepatocytes

1. In situ hepatic cannulation.
2. Perfusion with modified elasmobranch Ringer for 1 hour.
3. Perfusion with modified elasmobranch Ringer containing Clostridial collagenase for 1 hour.
4. Liver removed, finely dissected and filtered through 2 layers of gauze.
5. Resulting suspension spun at 80g at 4°C for 4 minutes.
6. Supernatant discarded, cells washed in calcium-free Ringer and recentrifuged as in 5. (repeated twice).
7. Final resuspension in a small volume of calcium-containing Ringer plus substrates for urea production.
8. Cells counted by haemocytometer and volume adjusted to give required number per ml.
9. Viability tested by exclusion of Trypan blue.

Assay of Urea Production

1. 1ml of final cell suspension (1 million cells) preincubated in glass vessel for 20 minutes at 10°C.
2. 0.5µCi [^{14}C]$NaHCO_3$ added to initiate reaction.
3. Vessels incubated for 2 hours at 10°C and $^{14}CO_2$ collected in hyamine solution.
4. Reaction stopped by addition of 100µl 60% perchloric acid.
5. Aliquot of reaction mixture incubated with urease for 2 hours and $^{14}CO_2$ collected in hyamine solution.
6. Hyamine solution added to scintillant and counted for carbon-14 on a liquid scintillation counter.

been pre-aerated with 95% O_2 /5% CO_2. After 1 hour the perfusate was changed to calcium-free Ringer containing clostridial collagenase (0.6 mg/ml) and the perfusion continued at 3 ml/min for another hour. The liver was then dissected out, cut up into small pieces and filtered through two consecutive layers of gauze (253µm and 73µm). The resulting suspension was centrifuged at 80g at 4 ^{o}C for 4 minutes. The supernatant was removed and discarded and the cells washed again in calcium-free Ringer. This process was repeated twice, the cells counted by haemocytometer and the final volume adjusted to give the required concentration of cells. Final resuspension was carried out in dogfish Ringer solution containing 2 mM calcium and substrates for urea production (1.0 mM serine, 0.5 mM glutamine and 0.5 mM aspartate). Viability was checked by exclusion of trypan blue and found to be better than 85%. Cells were used within two hours of isolation.

3.10.2 Assay of Urea Production

1 ml of final cell suspension, containing approximately a million cells (plus substrates) was added to a glass vessel and preincubated at 10 ^{o}C for 20 minutes (Figure 3.1). Each glass vessel contained a small plastic pot with 1ml of hyamine hydroxide and was sealed by a serological stopper. Incubations were initiated by the addition of Ringer containing substrates plus 0.5 µCi of [^{14}C] sodium bicarbonate (Amersham International PlC, Aylesbury, Bucks.) and the vessels placed in a shaking water bath for 2 hours at 10 ^{o}C. Reaction was stopped by

the addition of 100 µl of 60% perchloric acid. Any $^{14}CO_2$ given off was collected in the hyamine solution. An aliquot (1ml) of the incubation mixture was then incubated with 2.5 mg of urease in citric acid buffer for a further 2 hours and the $^{14}CO_2$ given off collected in fresh hyamine solution. Carbon-14 was then assayed by addition of scintillant to the hyamine and measured using a liquid scintillation counter. The amount of $^{14}CO_2$ formed was equivalent to the amount of urea produced. Equivalent incubations were carried out on both liver slices and liver homogenate preparations.

3.10.3 Assay of Carbamoyl Phosphate Synthetase III activity

The procedure followed was that of Ritter et al (1987) and is summarised in Figure 3.2. Liver tissue was removed and homogenised in 9 volumes of ice-cold homogenising solution (0.5 M sucrose, 0.15 M potassium chloride and 0.02 M Hepes, pH 7.5) for 15 seconds using a polytron (PT-1035, Kinematica, Switzerland). The homogenate was then filtered through two layers of gauze (253µm and 73µm) and centrifuged at 40g at 4 °C for 10 minutes. Centrifugation yielded three layers, a soft pellet, supernatant and an overlying "debris" layer which was mainly lipid. The supernatant was retained and the above procedure repeated. Following this, the supernatant was centrifuged at 800g at 4 °C for 10 minutes to sediment the nuclear fraction. The supernatant from this spin was then centrifuged at 15,000g at 4 °C for 10 minutes (J2-21 MIE, Beckmann Instruments

Figure 3.2

Fig. 3.2 Summary of Carbamoyl Phosphate Synthetase III Assay Procedure
(From Ritter, Smith and Campbell, 1987).

Fig. 3.2 Isolation of Mitochondria

1. Liver tissue homogenised for 15 seconds in 9 volumes of ice-cold homogenising fluid using a polytron.

2. Homogenate filtered through two layers of gauze and centrifuged at 40g at 4°C for 10 minutes.

3. Middle supernatant layer retained and re-centrifuged as 2.

4. Middle supernatant layer then centrifuged at 800g at 4°C for 10 minutes.

5. Supernatant spun at 15,000g at 4°C for 10 minutes.

6. Sedimented mitochondria resuspended in 10% of original homogenising volume and retained on ice until required

Variations:

1. Hand held glass homogeniser used instead of polytron.

2. Homogenates not filtered.

3. Pellets resuspended using a sonicator.

Assay of Carbamoyl Phosphate Synthetase III Activity

1. Aliquot of isolated mitochondria preincubated in 1ml of reaction mixture for 10 minutes.

2. Reaction initiated by addition of 10μCi ^{14}C-HCO_3 and incubation for 20 minutes at 30°C.

3. Reaction terminated by addition of 20% trichloroacetic acid.

4. Mixture gassed with CO_2 to remove unreacted ^{14}C-HCO_3.

5. Aliquots of reaction mixture added to scintillation fluid and counted by liquid scintillation for "fixed" carbon-14.

Variations:

1. Protein added: 0.4 - 10.0mg.

2. (i) Specific activity of ^{14}C-bicarbonate added: 0.02, 0.2 and 2.0μCi/μmol.
   (ii) Ornithinetranscarbamylase added: 1.8, 2.4, 3.0, 3.6, 4.2 amd 4.8 units/ml.
   (iii) Different reaction mixture compositions tested (Table 3.2).

Inc., California, U.S.A.) to prepare the mitochondrial fraction. The supernatant was discarded and the mitochondrial fraction resuspended in 10% of the original homogenising volume. Mitochondria were stored on ice and used within 2 hours of isolation, and an aliquot was taken for protein determination.

Carbamoyl phosphate synthetase III (CPS-III) activity was measured as the N-acetylglutamate-dependant fixation of acid-stable counts in the reaction mixture (Table 3.2). An aliquot of mitochondrial extract was pre-incubated in 1ml of reaction mixture for 10 minutes at 30 ^{o}C. The reaction was commenced by the addition of 10 µCi of [^{14}C] bicarbonate (0.02 µmole sodium [^{14}C] bicarbonate (Amersham) plus 5.0 µmole potassium bicarbonate to give a specific activity of 2 µCi/µmole) and the mixture incubated for 30 minutes at 30 ^{o}C. Reaction was stopped by the addition of 100 µl of trichloroacetic acid. Unreacted [^{14}C] bicarbonate was removed as $^{14}CO_2$ by gassing with CO_2 for 30 minutes, and portions of the mixture were then added to 3 ml scintillation fluid and counted for carbon-14. Calculation of endogenous activity was based on the specific radioactivity of the bicarbonate substrate and correction was made for non-specific fixation in the absence of N-acetylglutamate. The reaction pathway is stoichiometric and one µmole of bicarbonate fixed per hour at 30^{o}C is equivalent to one µmole of carbamoyl phosphate formed per hour at 30 ^{o}C.

Table 3.2

Table 3.2 Reaction Mixtures Used in the CPS III Assay

* 1 unit will convert 1µmole of phospho(enol) pyruvate to pyruvate per minute at pH 7.6 at 37°C.

+ 1 unit will form 1µmole of citrulline from ornithine and carbamoyl phosphate per minute are pH 8.5 at 37°C.

ECF, extracellular fluid; ICF, intracellular fluid; OTC, Ornithine Transcarbamylase

Table 3.2 Reaction Mixtures Used in the CPS III Assay

Reaction Mixture	Ritter et al (1987)	ECF-based	ICF-based
Reagent	Concentration (mmol/l)		
[14C]Na HCO_3	0.02	0.02	0.02
$KHCO_3$	5.0	5.0	5.0
$MgSO_4$	10.0	10.0	10.0
N-acetylglutamate	5.0	5.0	5.0
L-glutamine	10.0	10.0	10.0
L-ornithine	5.0	5.0	5.0
ATP	5.0	5.0	5.0
Phospho(enol) Pyruvate	2.5	2.5	2.5
Hepes	40.0	40.0	40.5
Pyruvate kinase	6.7 units/ml*	6.7 units/ml	6.7 units/ml
OTC	3.6 units/ml+	3.6 units/ml	3.6 units/ml
NaCl	-	240.0	6.0
KCl	-	7.0	40.0
$CaCl_2$	-	10.0	-
$MgCl_2$	-	4.9	-
$Na_2HPO_4 2H_2O$	-	0.5	-
Na_2SO_4	-	0.5	-
Urea	-	360.0	320.0
Trimethylamine-N-oxide	-	63.0	160.0
K_2HPO_4	-	-	40.0
$KHSO_4$	-	-	20.0
NaH_2PO_4	-	-	5.0
Sodium succinate	-	-	8.0
Sucrose	50.0	50.0	-
EGTA	-	-	0.4
EDTA	-	-	0.2
Osmolality (mOsm/Kg)	~200	~1100	~1000
pH	7.5	7.3	7.4

In addition to the above, certain modifications in procedure were carried out (Figure 3.2) and other reaction mixtures tested (Table 3.2).

3.10.4 Measurement of Citrulline Synthesis

The procedure used was that of Anderson and Casey (1984) and Anderson (1986) (Figure 3.3). All steps were carried out at 4 °C. Liver tissue was dissected out and minced with scissors. The tissue was then homogenised for 10 seconds in 9 volumes of ice-cold isolation medium (0.25M sucrose, 1mM EGTA, 0.3M urea, 0.15M TMAO, 0.15M potassium chloride and 20 mM Hepes, pH 7.5) using a polytron. The homogenate was then filtered through 2 layers of gauze (253 µm and 73 µm) and then centrifuged at 250g for 10 minutes. The supernatant was removed from the underlying pellet and overlying debris layers and then centrifuged at 8000g for 10 minutes to sediment the mitochondria. The sedimented mitochondria were washed carefully, the pellet resuspended in 9 volumes of isolation medium, and then centrifuged again at 8000g. The final supernatant was discarded and the mitochondrial pellet resuspended in 1 volume of isolation medium. Mitochondria were kept on ice, an aliquot taken for protein determination and were used within 2 hours of isolation.

Citrulline formation by isolated mitochondria was quantitatively determined by incubating mitochondria in a reaction mixture (Table 3.3) containing [^{14}C] bicarbonate (0.26 µCi/µmole) and ornithine and measuring the incorporation of radioactivity with time i.e. the

Figure 3.3

Fig. 3.3 Summary of Citrulline Synthesis Assay Procedure (From Anderson and Casey, 1984 and Anderson, 1986).

Fig. 3.3 Isolation of Mitochondria

1. Liver homogenised for 10 seconds in 9 volumes of homogenising solution using a polytron.

2. Homogenate filtered through 2 layers of gauze and centrifuged at 250g at 4°C for 10 minutes.

3. Middle supernatant layer retained and centrifuged at 8000g at 4°C for 10 minutes.

4. Sedimented mitochondria resuspended in homogenising solution and centrifuged as 3.

5. Sedimented mitochondria resuspended in 1 volume homogenising solution and stored on ice until required.

Variations tested similar to those in Figure 3.2.

Assay of Citrulline Synthesis

1. Aliquot of isolated mitochondria preincubated at 26°C for 5 minutes then added to reaction mixture to commence reaction.

2. Aliquots of reaction mixture removed after 30 minutes and added to 4M HCl in 0.1% Triton-X-100 to terminate reaction.

3. Unreacted ^{14}C-bicarbonate removed as $^{14}CO_2$ by addition of NH_4HCO_3 followed by brief and vigorous mixing (repeated twice).

4. 3ml scintillant added to each Aliquot of reaction mixture and Aliquots then counted for carbon-14 on a liquid scintillation analyser.

Table 3.3

Table 3.3 Reaction Mixtures Used in the Citrulline Synthesis Assay

Table 3.3 Reaction Mixtures Used in the Citrulline Synthesis Assay

Reaction Mixture	Anderson (1986)	ECF-based	ICF-based
Reagent	Concentration (mmol/l)		
$[^{14}C]KHCO_3$	6.0	6.0	6.0
$MgCl_2$	2.0	5.0	-
K_2HPO_4	4.0	-	40.0
KCl	80.0	7.0	50.0
Urea	60.0	360.0	320.0
Trimethylamine-N-oxide	30.0	63.0	160.0
EGTA	0.5	0.5	0.5
EDTA	0.4	0.4	0.4
Hepes	54.0	20.0	54.0
L-ornithine	5.0	5.0	5.0
L-glutamate	5.0	5.0	5.0
L-glutamine	5.0	5.0	5.0
Sucrose	50.0	50.0	50.0
Sodium succinate	8.0	8.0	8.0
$CaCl_2$	-	10.0	-
$NaH_2PO_4 2H_2O$	-	0.5	5.0
Na_2SO_4	-	0.5	-
NaCl	-	240.0	6.0
$MgSO_4$	-	5.0	8.0
$KHSO_4$	-	-	20.0
Osmolality (mOsm/Kg)	~475	~1100	~1050
pH	7.6	7.3	7.4

appearance of acid-stable radioactivity resulting from the formation of [^{14}C] carbamoyl phosphate from [^{14}C] bicarbonate, which then reacts with ornithine to give [^{14}C] citrulline. Acid-stable radioactivity not due to citrulline formation was determined by measuring radioactivity in reaction mixtures in which ornithine was absent.

1-1.5 mg of mitochondrial protein were pre-incubated at 26 °C for 5 minutes and then added to the reaction mixture (Table 3.3) to commence reaction (in a final volume of 0.5 ml). Aliquots (100 µl) were removed after 30 minutes and mixed with 50 µl of 4M hydrochloric acid, containing 0.1% triton X-100 (BDH), in the bottom of 6ml scintillation vials to stop reaction. Removal of all unreacted [^{14}C] bicarbonate (as $^{14}CO_2$) was accomplished by adding two 20 µl volumes (at 10 minute intervals) of 0.4M ammonium bicarbonate followed immediately by brief but vigorous mixing (Whirlimixer, Fisons, England). After an additional 20 minutes 0.2 ml of distilled water and 3ml of scintillant were added to each tube and the samples counted for carbon-14 on a liquid scintillation counter for 5 minutes (Figure 3.3). No acid-stable radioactivity was formed in the absence of mitochondria under these conditions. The reactions involved were stoichiometric and the rate of citrulline formation (µmole/h) could be calculated from the measured radioactivity and the known specific activity of the [^{14}C] bicarbonate added.

In addition to the above procedure, several modifications in the preparation of mitochondria were carried out (Figure 3.3). In the assay itself the amount of mitochondrial protein used was varied (0.3-3.0 mg) and also the specific activity of [^{14}C] bicarbonate was varied (0.26, 0.45 and 0.68 µCi/µmole). Other reaction mixtures were also tested (Table 3.3).

3.10.5 Measurement of Mitochondrial Respiration

The method used was that of Anderson (1986) and the isolation of mitochondria identical to the procedure described above. The respiration buffers used were identical to those described in Table 3.3 except that [^{14}C] potassium bicarbonate was replaced with potassium bicarbonate, L-glutamine and L-ornithine were 1mM and L-glutamate was 0.5 mM. In addition, during the experiments ADP was added to a final concentration of 1mM. As an added precaution to prevent bacterial contamination, the buffers used were aerated with filtered air (0.22 µm filter, Millipore U.K. Ltd.) and contained penicillin G and streptomycin (both 10 µg/ml) at concentrations which would not interfere with mitochondrial respiration (Ash, 1977). The rate of mitochondrial oxygen uptake was monitored using an oxygen monitor (Acid-Base Analyzer/Oxygen monitor, Radiometer, Copenhagen, Denmark) equipped with a Clark-type oxygen electrode (Radiometer), a 6ml stirred cell at 26°C and a Vitatron pen recorder. The rates of oxygen consumption were determined as follows. Aerated respiration buffer at 26 °C was pumped into the stirred

cell using a diaphragm pump (Micro Pump Series II, F.A. Hughes, Bournemouth). Once the solution in the chamber had been saturated with oxygen the inlet port was sealed and the pump switched off. The basal rate of oxygen consumption was then measured and was usually zero. Next, 8-12 mg of mitochondrial protein were added to the stirred chamber and the state 4 rate of oxygen consumption (respiration in the presence of 8mM succinate) determined over several minutes. ADP was then added to a final concentration of 1mM and the state 3 rate of respiration determined. Mitochondrial respiratory states are defined in Table 3.4. In this study the respiratory rate of primary interest was the state 3 rate of respiration. The procedures and definitions used were those by Estabrook (1967) and Chance and Williams (1959) except that state 4 is the rate before the addition of ADP rather than the rate following the expenditure of ADP, when a limiting amount of ADP is added to the system. The latter could not be accurately measured because the concentration of ADP required for maximal state 3 rates was higher than that characteristic of many other mitochondria and as a result oxygen would be depleted before all the ADP was used up, thus precluding the measurement of the rate of oxygen consumption following the expenditure of ADP (Anderson, 1986). State 4 is an anaerobic state, characterised by a low respiration rate, and requires that mitochondria be supplied with substrate (succinate) and without a phosphate acceptor (ADP). State 3 is an active state of rapid

Table 3.4

Table 3.4 Mitochondrial Respiratory States
(From Chance and Williams, 1959).
State 1 is an aerobic state, characterised by low respiration, in which the concentrations of both substrate and phosphate acceptor are low.
State 2 is a "starved" state in which ADP has been added in order to exhaust the endogenous substrate. Alternatively, mitochondria are freed of endogenous substrate by any process that will increase the endogenous ADP level sufficiently to promote rapid respiration.
State 3 is the "active" state of rapid respiration and phosphorylation which is achieved when adequate substrate and phosphate acceptor are present.
State 4 is an aerobic "resting" state characterised by low respiration even though substrate concentration is high. It is established when carefully prepared mitochondria are supplied with adequate substrate but without phosphate receptor.
State 5 is characterised by zero respiration. Oxygen is lacking and all the components associated with the respiratory pathway are reduced. The ADP level is consequently high in state 5.

Table 3.4 Mitochondrial Respiratory States
(From Chance and Williams, 1959)

State	O_2	ADP Level	Substrate Level	Respiration Rate	Rate-limiting Substance
1	>0	low	low	slow	ADP
2	>0	high	~0	slow	substrate
3	>0	high	high	fast	respiratory chain
4	>0	low	high	slow	ADP
5	0	high	high	0	oxygen

respiration and phosphorylation, with adequate supplies of substrate and phosphate acceptor. The respiratory control ratio, the ratio of state 3: state 4, is a measure of mitochondrial viability and indicates the ability of mitochondria to maximally increase their respiratory rates in the presence of substrate and phosphate acceptor i.e. it gives a measure of how well "coupled" oxidation and phosphorylation are in a given mitochondrial preparation.

3.11 Isolated Perifused Interrenal System

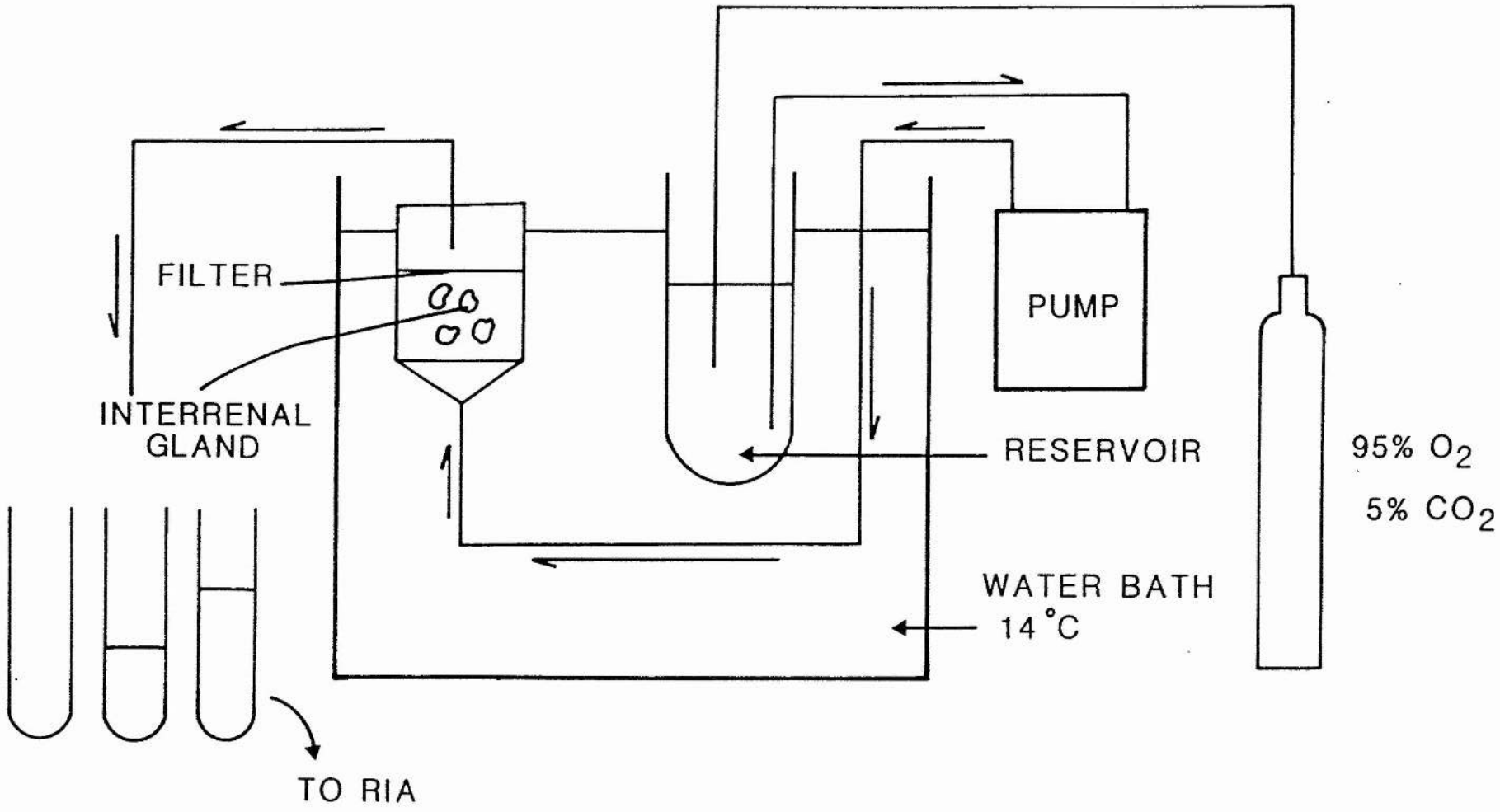
The perifusion system used is shown in Figure 3.4.

3.11.1 Apparatus

Perifusion chambers were constructed from modified 5ml plastic syringes and sealed at the top with air tight plastic lids. The inlet port of the chamber was formed by placing a needle on the end of the modified syringe and the outlet port was formed by inserting a syringe needle, separated from its plastic base, through the lid of the chamber. Three plastic rings formed the inner chamber and a 1mm nylon mesh was placed across each end to form a cylinder in which interrenal tissue could be placed. Perifusion medium could then be introduced to the chamber via the inlet needle, passed across the gland tissue and removed via the exit port. A peristaltic pump (Watson-Marlow 502S, Belmont Instruments, Glasgow) removed incubation media from a reservoir, maintained at 12-14 °C, via cannula tubing (i.d.= 0.76 mm; Portland Plastics,Kent). The perifusion medium was then pumped at a rate of 0.5 ±

Figure 3.4

Fig. 3.4 Schematic Diagram of the Isolated Perifused Interrenal Gland Apparatus (From O'Toole, 1987).


FILTER
INTERRENAL GLAND
PUMP
RESERVOIR
95% O_2
5% CO_2
WATER BATH
14 °C
TO RIA

0.05 ml/min into the perifusion chamber, across the interrenal tissue and through the outlet port to be collected, using a fraction collector (Ultrorac 7000, L.K.B., Bromma, Sweden), every 15 minutes into glass test tubes. 200µl of 2M sulphuric acid were then added to each tube and the mixture vortexed. Samples were then incubated for 2 hours at 80 ^{o}C to facilitate the quantitative conversion of 1α-OH-B to 1-dehydrocorticosterone(Kime, 1977). Following this, the samples were extracted, in preparation for assay, using SEP PAK C18 cartridges as described previously.

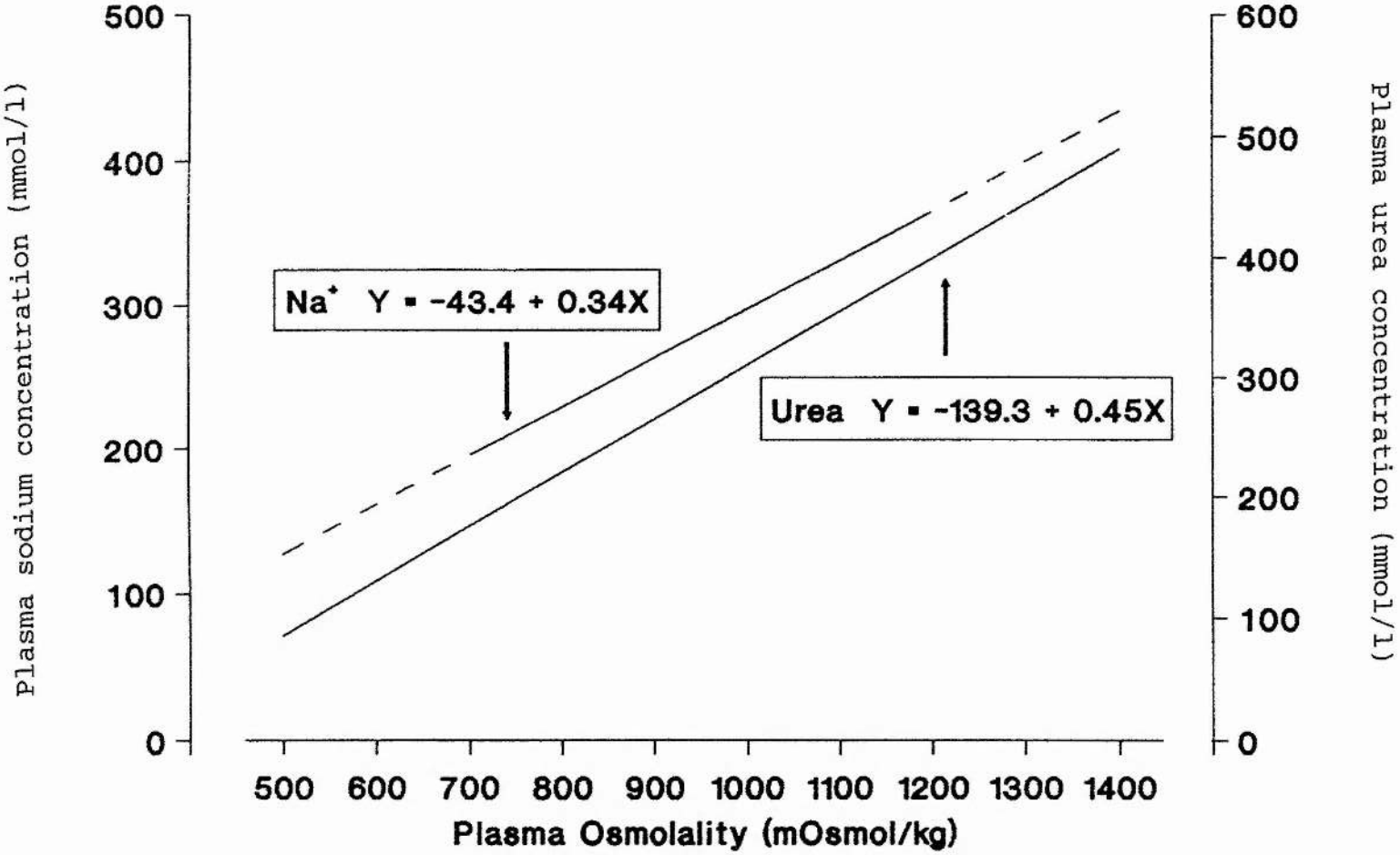
3.11.2 Preparation of Perifusion Medium

The perifusion medium used consisted of the basic elasmobranch Ringer solution (Appendix 2.). 24 hours prior to experiment, 1ml blood samples were taken from test animals and plasma osmolality measured using a freezing point depression osmometer (Camlab). Ringer solution was then made up to the required osmolality by adjusting the concentrations of sodium and urea, the two principal osmolytes manipulated by elasmobranchs to maintain an iso-osmotic plasma. The variations in sodium and urea concentrations were carried out using geometric mean regression lines for plasma osmolality against plasma sodium and urea concentrations, respectively (Figure 3.5.). Geometric mean regression equations were calculated using data obtained by Hazon (1982) and from HPD fish used in this study. Plasma urea concentration varies proportionately with changes in plasma osmolality when

Figure 3.5

Fig. 3.5 Regression Lines for Plasma Osmolality Against Plasma Sodium and Urea Concentrations

Geometric mean regression lines and equations for plasma osmolality against plasma sodium and plasma urea concentrations, respectively. Changes in plasma sodium concentration are only directly proportional to changes in plasma osmolality over the range 700 mOsmol kg^{-1} to 1200 mOsmol kg^{-1}. The dashed lines represent the ranges of plasma osmolality where plasma sodium concentration appears to be regulated and does not fit the regression equation. These regions were not used for the prediction of sodium concentrations.


Plasma sodium concentration (mmol/l)
Plasma urea concentration (mmol/l)
Na+ Y = -43.4 + 0.34X
Urea Y = -139.3 + 0.45X
500
400
300
200
100
0
600
500
400
300
200
100
0
500 600 700 800 900 1000 1100 1200 1300 1400
Plasma Osmolality (mOsmol/kg)

dogfish are adapted from 50% - 130% seawater (Hazon and Henderson, 1984) and data from this range was used to calculate the geometric mean regression equation for urea, which was $Y = -139.3 + 0.45X$. Plasma sodium concentration varies linearly with changes in plasma osmolality, during adaptation, between 70 and 120% seawater, beneath or above which sodium regulation occurs around a new lower or upper limit, respectively (Hazon and Henderson, 1984). Consequently, only plasma sodium data from the linear variation range was used in the calculation of the geometric mean regression equation, which was $Y = -43.4 + 0.34X$.

Linear (or ordinary) regression assumes that the independent (x-axis) variable is measured without error. When both variables are measured, geometric mean regression is useful because it minimises the errors from both sets of variables and yields a truer estimate of central tendency (Ricker, 1973; Sokal and Rohlf, 1981).

3.11.3 Perifusion Protocol

Dogfish weighing 800-1000g were sacrificed by a blow to the head and severing of the spinal cord. Interrenal glands were removed and immediately placed in ice-cold elasmobranch Ringer solution prior to weighing. Each gland was then diced into approximately 1mm fragments and placed between the two mesh layers in the inner perifusion chamber. The glands were covered with Ringer solution and the chamber sealed prior to connection to the pump. Special care was taken to avoid trapping air bubbles in the

system. For the first two hours of each experiment the incubation medium was run to waste in order to attain a steady basal secretory rate.

Each test substance was dissolved in perifusion medium immediately prior to use and then administered in a 3 hour dose-response cycle. In experiments using calcium-free medium, 0.4mM EGTA was added to the Ringer solution. This comprised a 1 hour control period, a 15 minute stimulation period during which the test substance was applied, and a further 1 hour and 45 minute collection period to permit a full response to the test substance and subsequent return to a steady basal level. The perifusion experiments carried out in this study are listed in Table 3.5.

3.11.4 Analysis of Results

For the dietary and protein adaptation data, overall differences were assessed using analysis of variance and specific statistical differences between groups determined using the Students t-test. Perifusion results were calculated following the method established in the literature (Perroteau et al, 1984; Lihrmann et al, 1985; Feuilloley et al, 1988; Leboulenger et al, 1988). Results used were peak responses, expressed as the percentage increase above the basal secretory rate. Basal secretory rate was taken as the mean of four 15 minute collection periods immediately prior to the addition of test substance and is expressed in fmol/mg of tissue per 15 minutes. All results are means +/- standard error of the mean (S.E.M.). Where a time course has been shown, results are expressed

Table 3.5

Table 3.5 Summary of Perifusion Experiments

Table 3.5 Summary of Perifusion Experiments

1. Porcine ACTH (0.01, 0.1, 1.0 & 10μM)
2. Dibutyryl cAMP (0.01 & 1nM, 0.1 & 10μM)
3. Comparison of cAMP (10μM) and forskolin (1μM)
4. Effect of cholera toxin (2 and 4μg/ml) on: (i) basal secretory rate (ii) response to 0.1μM porcine ACTH (4μg/ml only)
5. Effect of Ca2+-free Ringer on: (i) basal secretory rate (ii) response to 0.1μM porcine ACTH
6. Effect of 50μM dantrolene on: (i) basal secretory rate (ii) response to 0.1μM porcine ACTH
7. Effect of 10μM verapamil on: (i) basal secretory rate (ii) response to 0.1μM porcine ACTH
8. Val5-AII (0.01, 0.1 & 1.0μM)
9. Comparison of Val^5- and Ile^5-AII (both 0.1μM)
10. Effect of Ca^{2+}-free Ringer on response to 0.1μM Ile^5-AII
11. Effect of 50μM dantrolene on response to 0.1μM Ile^5-AII
12. Effect of 10μM verapamil on response to 0.1μM Ile^5-AII
13. Effect of Ca^{2+}-ionophore, A23187 (10μM)
14. Comparison of ANP and AVT (both 0.1μM)
15. Dibutyryl cGMP (0.01 & 1nM, 0.1 & 10μM)
16. Potassium (12,18, 28 & 40mM)
17. Sodium (320, 360 & 400mM)

as a percentage of basal secretory rate for clarity of presentation. Statistical significance was determined using the Student's t-test.

4. RESULTS

4.1 Radioimmunoassay of 1α-hydroxycorticosterone

Figure 4.1 shows an antibody dilution curve for 1-dehydrocorticosterone. The relationship is typically sigmoidal and an antibody dilution of 1:1500 was chosen for the assay. This dilution gave a B_o of approximately 40% and differential binding of 34% for standards ranging from 0-4ng, and a typical standard curve is shown in Figure 4.2.

The assay sensitivity was 0.065 ng, the inter-assay variation 8.26 ± 0.21% (mean ± S.E.M., n=20) and the intra-assay variation 5.41 ± 0.43% (mean ± S.E.M., n=22).

The antibody used was originally raised to corticosterone and its cross reactivities are given in Table 4.1. In view of the potentially high cross reactivities of the antibody to other steroids, plasma and perifusion sample extracts were assayed separately, using specific antibodies, for the presence of corticosterone, deoxycorticosterone and cortisol. Once the values obtained had been corrected for cross reactivity with 1α-OH-B in their respective assays, deoxycorticosterone represented a maximal 4.9% of the total steroid in the perifusion samples and a maximal 2.4% of the total steroid in the plasma samples and corticosterone represented a maximal 5.1% of the total steroid in the perifusion samples. Cortisol was not detected in plasma or perifusion samples. The errors associated with cross reactivity of 1-dehydrocorticosterone anti-serum with other corticosteroids, present in the plasma and perifusion samples in small quantities, are less

Figure 4.1

Fig. 4.1 Binding of ^{3}H-1-dehydrocorticosterone at Different Dilutions of Antibody
Antibody dilution (abscissa) is plotted against percentage binding (ordinate). Each point is the mean of four determinations.

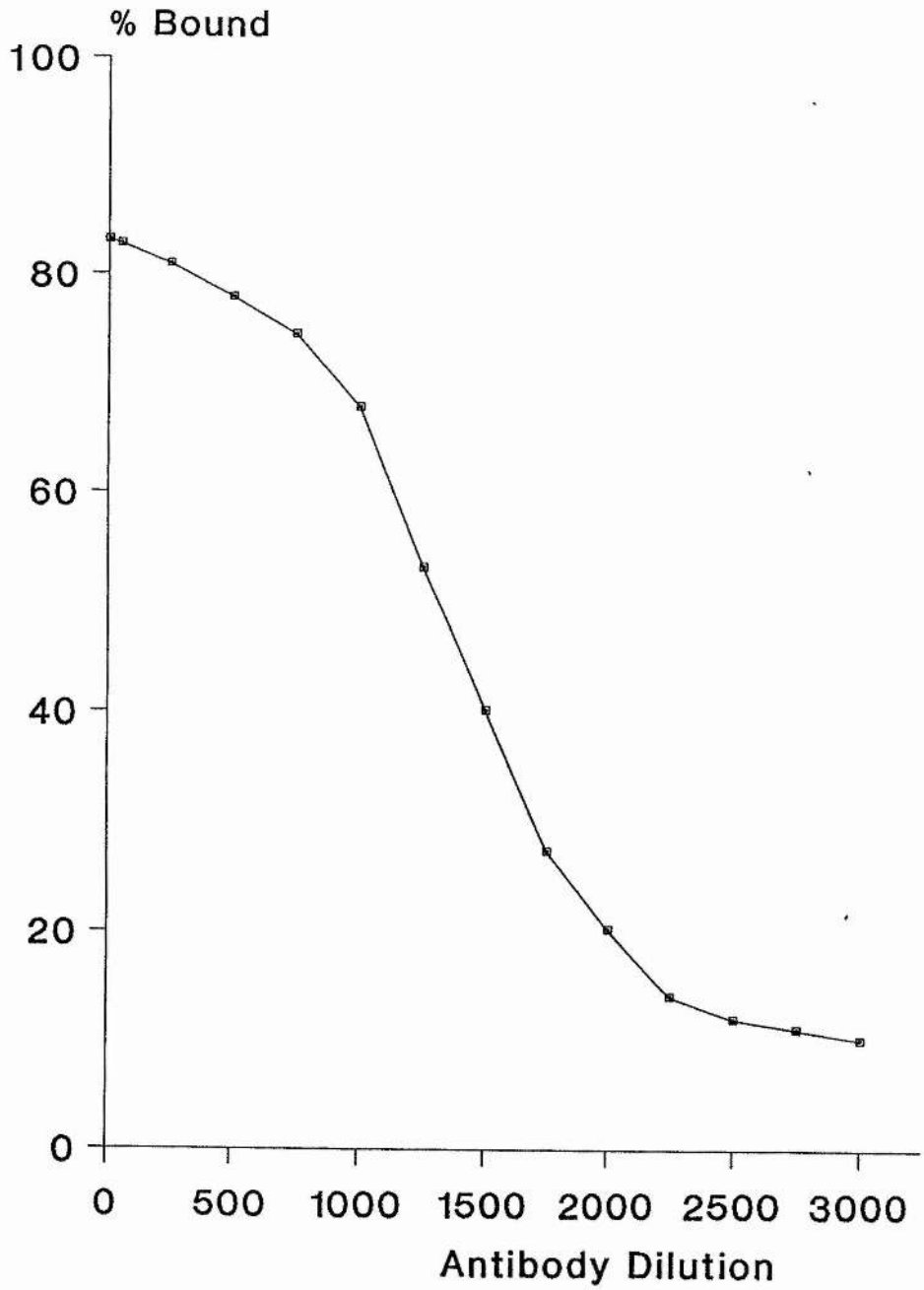

% Bound
100
80
60
40
20
0
0
500
1000
1500
2000
2500
3000
Antibody Dilution

Figure 4.2

Fig. 4.2 Standard Curve for the Radioimmunoassay of 1-dehydrocorticosterone
The amount of 1-dehydrocorticosterone (ng) is plotted on the abscissa against percentage binding on the ordinate. Each point represents the mean ± S.E.M. of 40 replicates.

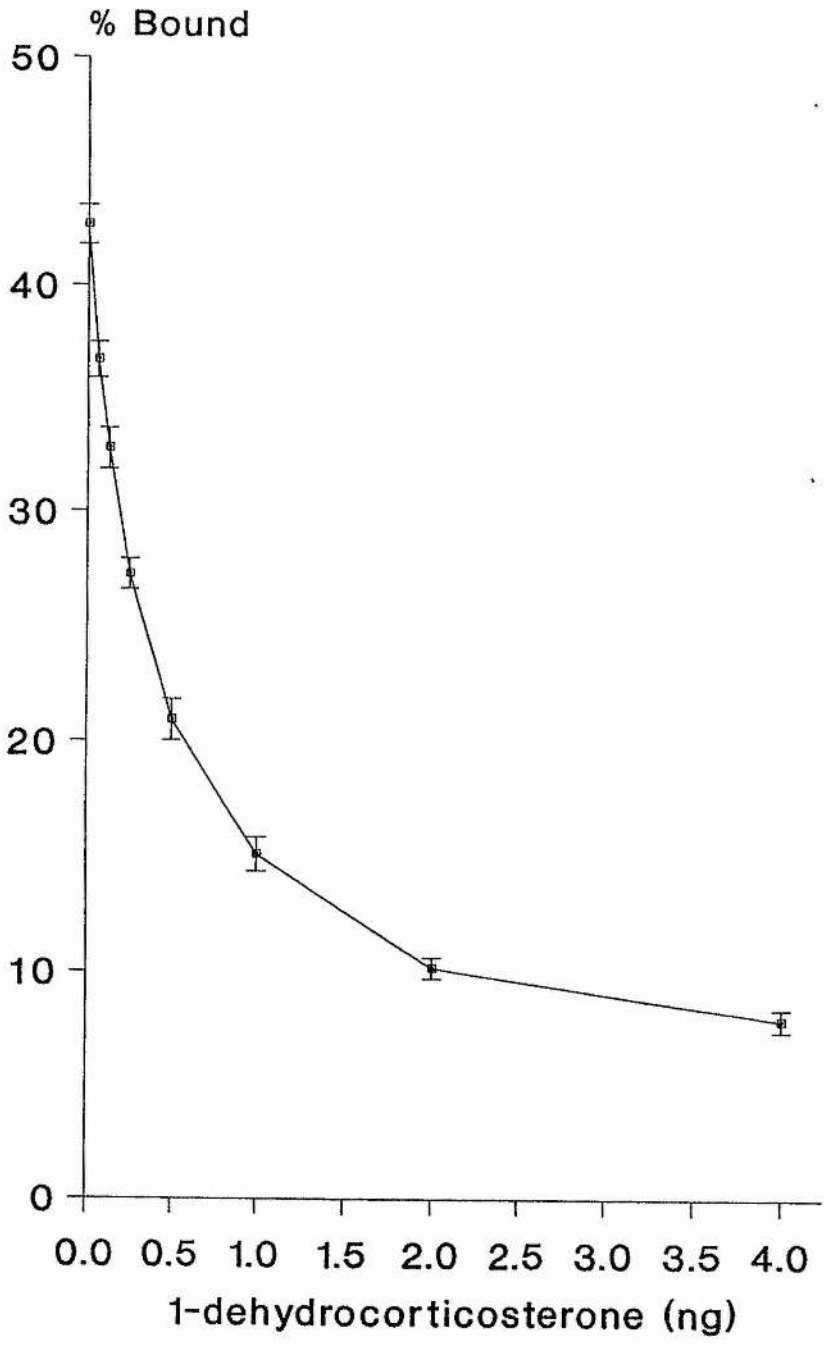

% Bound
50
40
30
20
10
0
0.0 0.5 1.0 1.5 2.0 2.5 3.0 3.5 4.0
1-dehydrocorticosterone (ng)

Table 4.1

Table 4.1 Cross Reactivities of Antiserum Used in the 1-dehydrocorticosterone Assay
* Values are based on cross reactivity to 1-dehydrocorticosterone.

Table 4.1 Cross Reactivites of Antiserum Used in the 1-dehydrocorticosterone Assay

Steroid	% Cross-Reactivity*
Corticosterone	124%
Cortisol	98%
Deoxycorticosterone	92%
Progesterone	70%
Aldosterone	42%
1α-hydroxycorticosterone	2.3%
1-dehydrocorticosterone	100.0%

than the normal intra-assay variation and therefore do not appreciably diminish assay performance.

4.2 Assessment of C18 SEP PAKs for use in steroid extraction

4.2.1 Extraction of plasma samples

Table 4.2a shows the relative proportions of ^{3}H-1α-OH-B extracted during each phase of the elution procedure. Little steroid was lost in the washing step (water phase) with the majority of steroid being recovered in the first 2ml of methanol elution and quantitatively all of the extractable steroid recovered after elution with 3ml of methanol. The elution rate used for this initial experiment was 1ml min^{-1} and was subsequently found not to be optimal. Table 4.2b shows the effects of elution rate and pH on the extraction of ^{3}H-1α-OH-B from dogfish plasma.

Percentage recovery of ^{3}H-1α-OH-B from acidified plasma did not represent an improvement over recovery from plasma at normal pH (Table 4.2b). Acidification of mammalian plasma is necessary for quantitative steroid extraction using C18 SEP PAK cartridges as it facilitates the dissociation of steroids from their binding proteins. Elasmobranch plasma has been shown not to possess significant amounts of steroid-binding proteins (Idler, Freeman and Truscott, 1967; Idler and Freeman, 1968) and the absence of a pH effect on the percentage recovery of ^{3}H-1α-OH-B provides further indirect confirmation of this.

An elution rate of 0.3ml min^{-1} proved to be optimal for ^{3}H-1α-OH-B extraction from plasma and was subsequently

Table 4.2

Table 4.2a Extraction of Plasma Samples
Extraction of ^{3}H-1α-hydroxycorticosterone from dogfish plasma using SEP PAK C18 cartridges. Elution rate 1mlmin^{-1}. Values are means ± S.E.M. of 16 replicates.

Table 4.2b Effect of pH and Elution Rate on the Extraction of Plasma Samples
Values are means ± S.E.M. of 16 replicates.

Table 4.2a Extraction of Plasma Samples

% Recovery	Mean ± S.E.M.
In water phase	0.9 ± 0.1
In 1st 2ml methanol	78.2 ± 7.3
In 3rd ml methanol	3.8 ± 0.6
In 4th ml methanol	not detectable
Total recovery (%)	82.9 ± 5.6

Table 4.2b Effect of pH and Elution rate on the Extraction of Plasma Samples

Rate of Elution (ml/min)	% Recovery (Mean ± S.E.M.) pH7	pH4
0.3	91.2 ± 1.2	92.2 ± 1.5
1.0	83.4 ± 2.8	83.2 ± 2.0
2.0	81.3 ± 3.6	80.2 ± 1.7
4.0	76.0 ± 0.4	76.9 ± 0.5

used for all plasma steroid extractions. The subsequent mean (± S.E.M.) extraction of 1α-OH-B from dogfish plasma was found to be 90.6 ± 0.3% (n=200).

4.2.2 Extraction of perifusion samples

The relative proportions of ^{3}H-1-dehydrocorticosterone extracted during each phase of the elution procedure are shown in Table 4.3a. Little steroid is lost during the washing step (water phase), with the majority of steroid being recovered in the first 2ml of methanol elution and quantitatively all of the extractable steroid recovered after elution with 3ml of methanol. The elution rate used for this experiment was 1ml min^{-1} and was subsequently found not to be optimal.

Table 4.3b shows the effect of elution rate on the excretion of ^{3}H-1-dehydrocorticosterone using SEP PAK C18 cartridges. The optimal rate of elution for the extraction of ^{3}H-1-dehydrocorticosterone was found to be 4ml min^{-1} and was subsequently used for all perifusion sample extractions. The subsequent mean extraction of 1-dehydrocorticosterone was found to be greater than 99% (n=2300).

The use of SEP PAK C18 cartridges for the extraction of steroids from dogfish plasma and perifusion medium has been a considerable improvement on the other methods used which produced a maximal extraction of 80% and took 24 hours to complete.

Table 4.3

Table 4.3a Extraction of Perifusion Samples
Extraction of ^{3}H-1-dehydrocorticosterone from perifusion media using SEP PAK C18 cartridges. Elution rate 1mlmin^{-1}. Values are means ± S.E.M. of 16 replicates.

Table 4.3b Effect of Elution Rate on the Extraction of Perifusion Samples
Values are means ± S.E.M. of 16 replicates.

Table 4.3a Extraction of Perifusion Samples

% Recovery	Mean ± S.E.M.
In water phase	0.4 ± 0.1
In 1st 2ml methanol	81.5 ± 2.6
In 3rd ml methanol	2.4 ± 0.3
In 4th ml methanol	not detectable
Total recovery (%)	84.3 ± 3.3

Table 4.3b Effect of Elution rate on the Extraction of Perifusion Samples

Rate of Elution ml min^{-1}	% Recovery (Mean ± S.E.M.)
1.0	82.2 ± 0.5
2.0	93.0 ± 1.4
4.0	100.1 ± 2.2
6.0	85.9 ± 1.0

4.3 Metabolic clearance rate

The constant infusion technique has proved to be suitable for measuring MCR in dogfish. Urinary production rate methods have proved unsuitable for clearance rate measurements in Scyliorhinus canicula because collecting urine at stable and reliable rates has proved difficult in fish weighing less than 1Kg. In male fish the urinary and reproductive systems share a common duct rendering the collection of uncontaminated urine impossible. In female fish the complexity of the urinary ducts and the narrow urinary papilla have rendered reliable and stable urine collections difficult. Thus, the MCR determinations carried out were measured by blood analysis only.

4.3.1 Constant infusion techniques

a) 1α-hydroxycorticosterone

Figure 4.3a represents a typical ^{3}H-1α-OH-B infusion experiment. Investigation of several techniques led to the use of a priming dose of 5μCi ^{3}H-1α-OH-B followed by a constant infusion of 0.5μCi h^{-1} at a rate of 6μl min^{-1}. This regime led to an equilibration time of 5 to 6 hours. During the infusion the endogenous plasma 1α-OH-B concentration did not change.

b) Urea

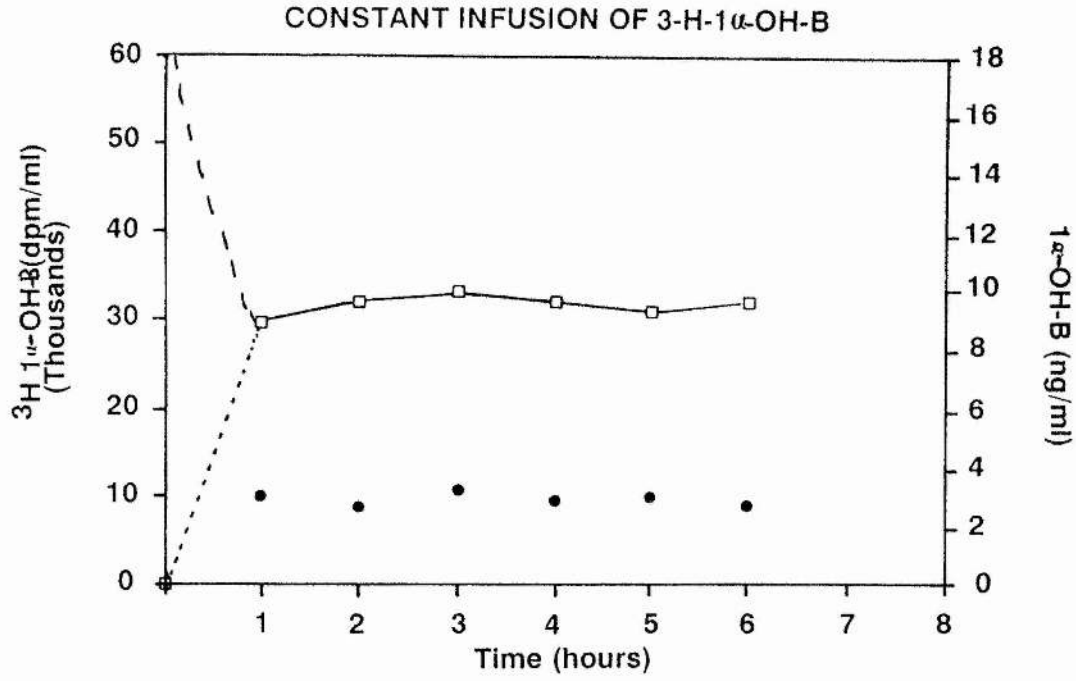
A typical [^{14}C] urea infusion experiment is shown in Figure 4.3b. A priming dose of 10μCi [^{14}C] urea, followed by a constant infusion of 1μCi h^{-1} at a rate of 6μl min^{-1} was used. The endogenous plasma urea concentration did not change during the infusion.

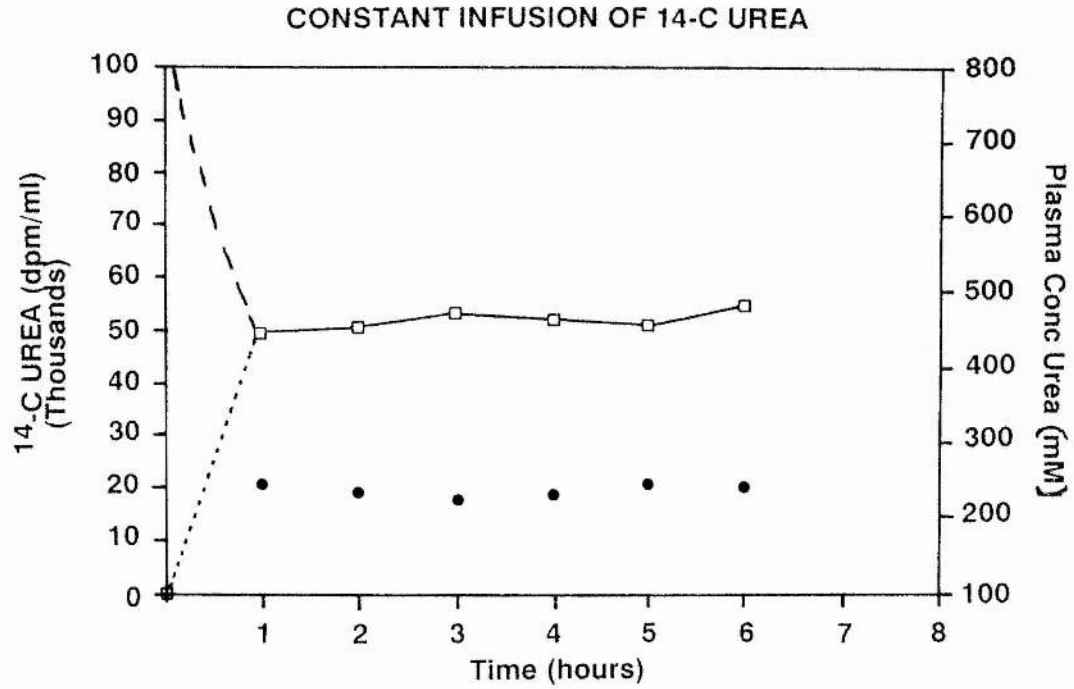
Figure 4.3

Fig. 4.3 Constant Isotopic Infusions

a) Typical experiment to determine the time to achieve a "steady state" of radioactivity in plasma during continuous infusion of tritiated 1α-hydroxycorticosterone (^{3}H-1α-OH-B). Following an injection of 5μCi, an infusion (0.5μCi h^{-1}) was begun at time zero, and blood samples were withdrawn at intervals for six hours. Solid circles show endogenous 1α-OH-B concentration (ng ml^{-1}) and open squares the plasma concentration of tritium (dpm ml^{-1}). The dashed and dotted lines represent the estimated contributions to plasma radioactivity made by the constant infusion and injection of tracer, respectively, during the first, pre-equilibration hour of experiment.

b) Typical experiment to determine the time to achieve a "steady state" of radioactivity in plasma during the continuous infusion of [^{14}C]-urea. Following an injection of 10μCi, an infusion (1.0μCi h^{-1}) was begun at time zero and blood samples were withdrawn at intervals for six hours. Solid circles represent endogenous plasma urea concentration (mM) and open squares the plasma concentration of carbon-14 (dpm ml^{-1}). The dashed and dotted lines represent the estimated contributions to plasma radioactivity made by the constant infusion and injection of tracer, respectively, during the first, pre-equilibration hour of experiment.


CONSTANT INFUSION OF 3-H-1α-OH-B
^{3}H 1α-OH-B (dpm/ml) (Thousands)
1α-OH-B (ng/ml)
Time (hours)
0
10
20
30
40
50
60
0
2
4
6
8
10
12
14
16
18
1
2
3
4
5
6
7
8


CONSTANT INFUSION OF 14-C UREA
^{14}-C UREA (dpm/ml) (Thousands)
Plasma Conc Urea (mM)
Time (hours)
0
10
20
30
40
50
60
70
80
90
100
100
200
300
400
500
600
700
800
1
2
3
4
5
6
7
8

4.4 The effects of high and low protein diets and environmental salinity on plasma composition and steroid dynamics

4.4.1 Feeding regimes

Initial studies concentrated on determining a maintenance feeding regime. To maintain dogfish body weight over a period of 30 days a feeding regime of 2.5g diet per kg fish every second day was estimated. This proved successful in that the fish readily ate both diets at this frequency of feeding and both groups maintained body weight. For the high protein diet (HPD) fish initial weight was 928 ± 7.2g before adaptation and 914 ± 10.2g at the end. For low protein diet (LPD) fish initial weight was 950 ± 8g before and 944 ± 10g after dietary adaptation (n=20 in both cases).

4.4.2 Changes in plasma osmolality

In both dietary groups plasma osmolality varied proportionately with changes in seawater, remaining iso- or slightly hyper-osmotic in both 50% and 130% seawater (Fig. 4.4). The plasma osmolality in 130% seawater was significantly higher ($p<0.005$) than that in 100% seawater and significantly lower in 50% seawater ($p<0.005$).

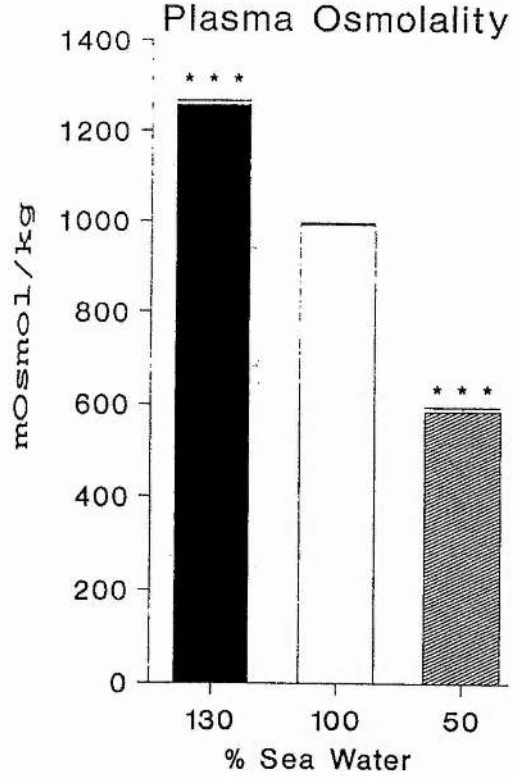
4.4.3 Changes in plasma electrolyte concentrations

The changes in plasma electrolyte concentrations of HPD fish are shown in Figure 4.5. Both plasma sodium and plasma chloride concentrations varied proportionately with changes in the environmental seawater, being significantly higher in fish adapted to 130% seawater ($p<0.005$ and

Figure 4.4

Fig. 4.4 Changes in Plasma Osmolality
Plasma osmolality (mOsmol/Kg) for (a) high protein diet fish and (b) low protein diet fish adapted to 100% seawater (clear bars), 130% seawater (solid bars) and 50% (hatched bars). Results are means ± S.E.M. Number of animals:
(a) for 130% seawater, n=6; for 100% seawater, n=11; and for 50% seawater, n=6.
(b) for 130% seawater, n=7; for 100% seawater, n=11; and for 50% seawater, n=6.
*** indicates statistically significant differences at $p<0.005$ (Student's t-test) compared with values obtained in 100% seawater.

a)


Plasma Osmolality
1400
1200
1000
800
600
400
200
0
mOsmol/kg
* * *
* * *
130
100
50
% Sea Water

b)

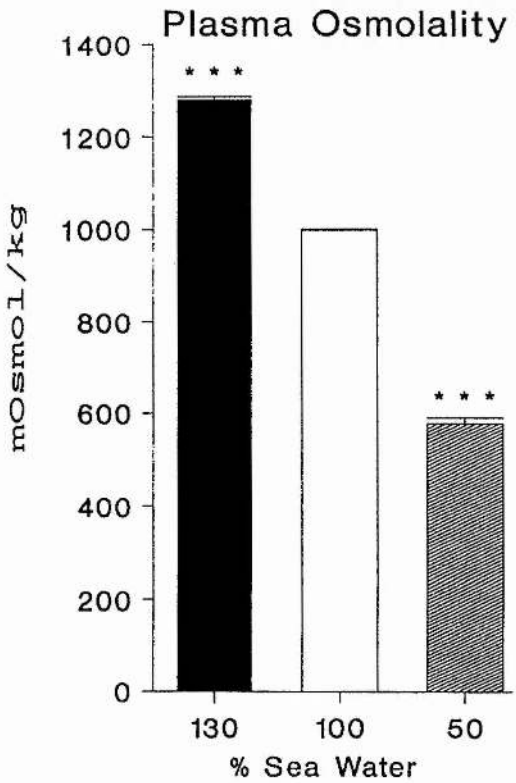
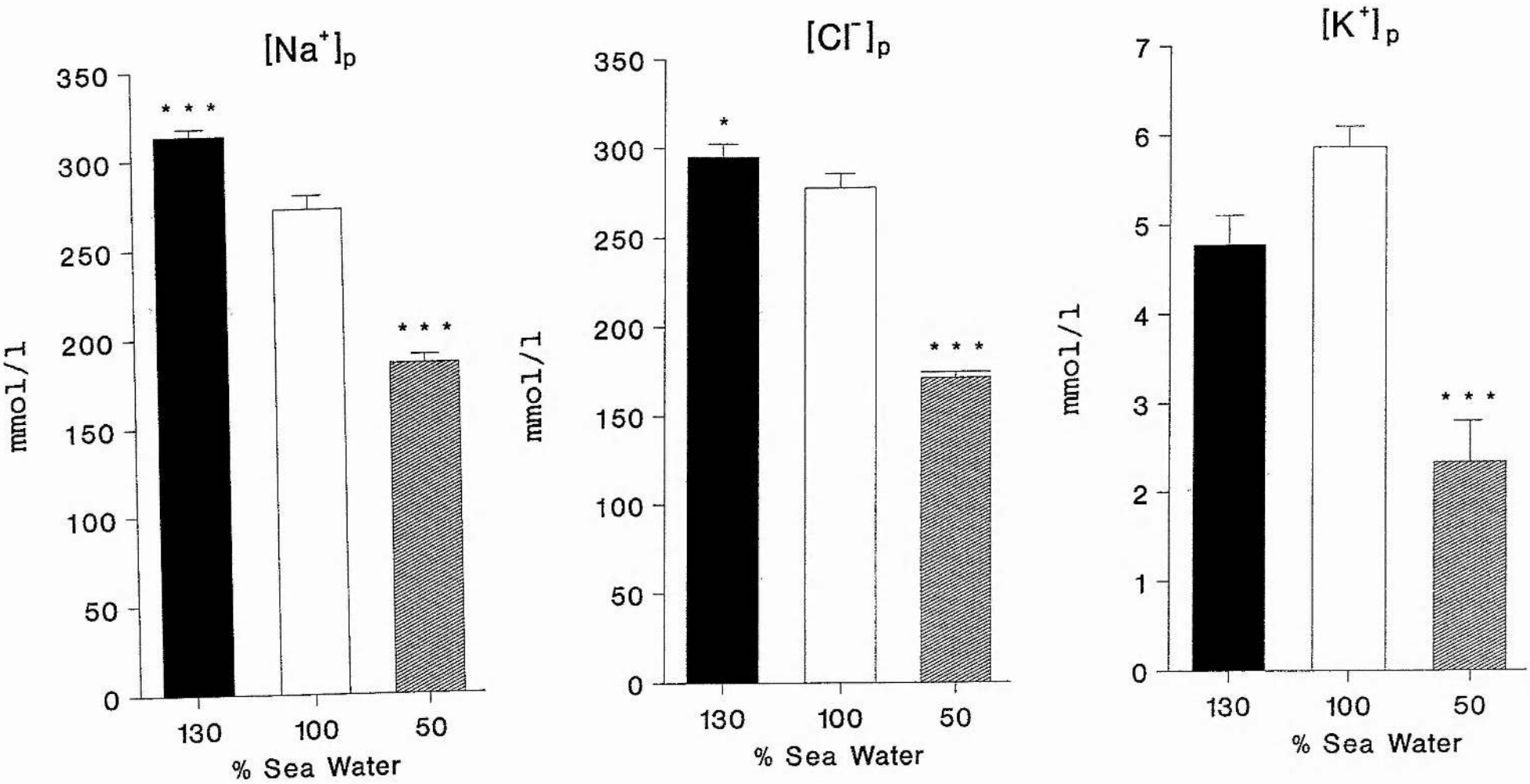

Plasma Osmolality
1400
1200
1000
800
600
400
200
0
mOsmol/kg
* * *
* * *
130
100
50
% Sea Water

Figure 4.5

Fig. 4.5 Changes in Plasma Electrolytes in High Protein Diet Fish

Plasma Na^+, Cl^- and K^+ concentrations (all mmol/l) in dogfish on a high protein diet adapted to normal seawater (clear bars), 130% seawater (solid bars) and 50% seawater (hatched bars). Results are the means ± S.E.M. Number of animals: for 130% seawater, n=9; for 100% seawater, n=10; and for 50% seawater, n=10. *** and * indicate statistically significant differences at $p<0.005$ and $p<0.05$, respectively (Student's t-test) from values seen in 100% seawater.


[Na+]p
mmol/l
350
300
250
200
150
100
50
0
* * *
* * *
130
100
50
% Sea Water
[Cl-]p
mmol/l
350
300
250
200
150
100
50
0
*
* * *
130
100
50
% Sea Water
[K+]p
mmol/l
7
6
5
4
3
2
1
0
* * *
130
100
50
% Sea Water

$p<0.05$, respectively) and lower in fish adapted to 50% seawater ($p<0.005$ in both cases). The overall effect on plasma osmolality was to maintain it iso-osmotic with the particular environmental seawater. Plasma potassium concentrations were significantly reduced in 50% seawater but appeared unchanged in 130% seawater.

Figure 4.6 shows the changes in electrolyte content of fish on a LPD in 130% and 50% seawater. Qualitatively the changes observed were similar to those observed for the HPD fish.

Electrolyte concentrations were similar in both groups with the exception of plasma Na^+ and Cl^- concentrations which were significantly higher ($p<0.05$, t-test) in fish on a LPD in 130% seawater than in the corresponding group of HPD fish. Subsequent dissection of these animals revealed the presence of seawater in the guts of the LPD group but not in the guts of the HPD group in 130% seawater, indicating that the LPD animals had been drinking (Hazon, Balment, Perrott and O'Toole, 1989) to survive the osmotic stress and would explain the extremely high plasma Na^+ and Cl^- concentrations in these animals.

4.4.4 Urea dynamics

Figure 4.7 shows the urea dynamics of fish on a HPD. Plasma urea concentration varied in direct proportion to changes in the environmental osmolality, in order to maintain the plasma iso-osmotic with the environmental seawater. In 130% seawater the increased plasma urea concentration ($p<0.01$) was achieved by decreasing MCR ($p<0.005$). In 50% seawater the decrease in plasma urea

Figure 4.6

Fig. 4.6 Changes in Plasma Electrolytes in Low Protein Diet Fish

Plasma Na^+, Cl^- and K^+ concentrations (all mmol/l) in dogfish on a low protein diet adapted to normal seawater (clear bars), 130% seawater (solid bars) and 50% seawater (hatched bars). Results are means ± S.E.M. Number of animals = 10 in each case.
*** and ** indicate statistically significant differences at $p<0.005$ and $p<0.05$, respectively (Student's t-test) from values seen in 100% seawater.

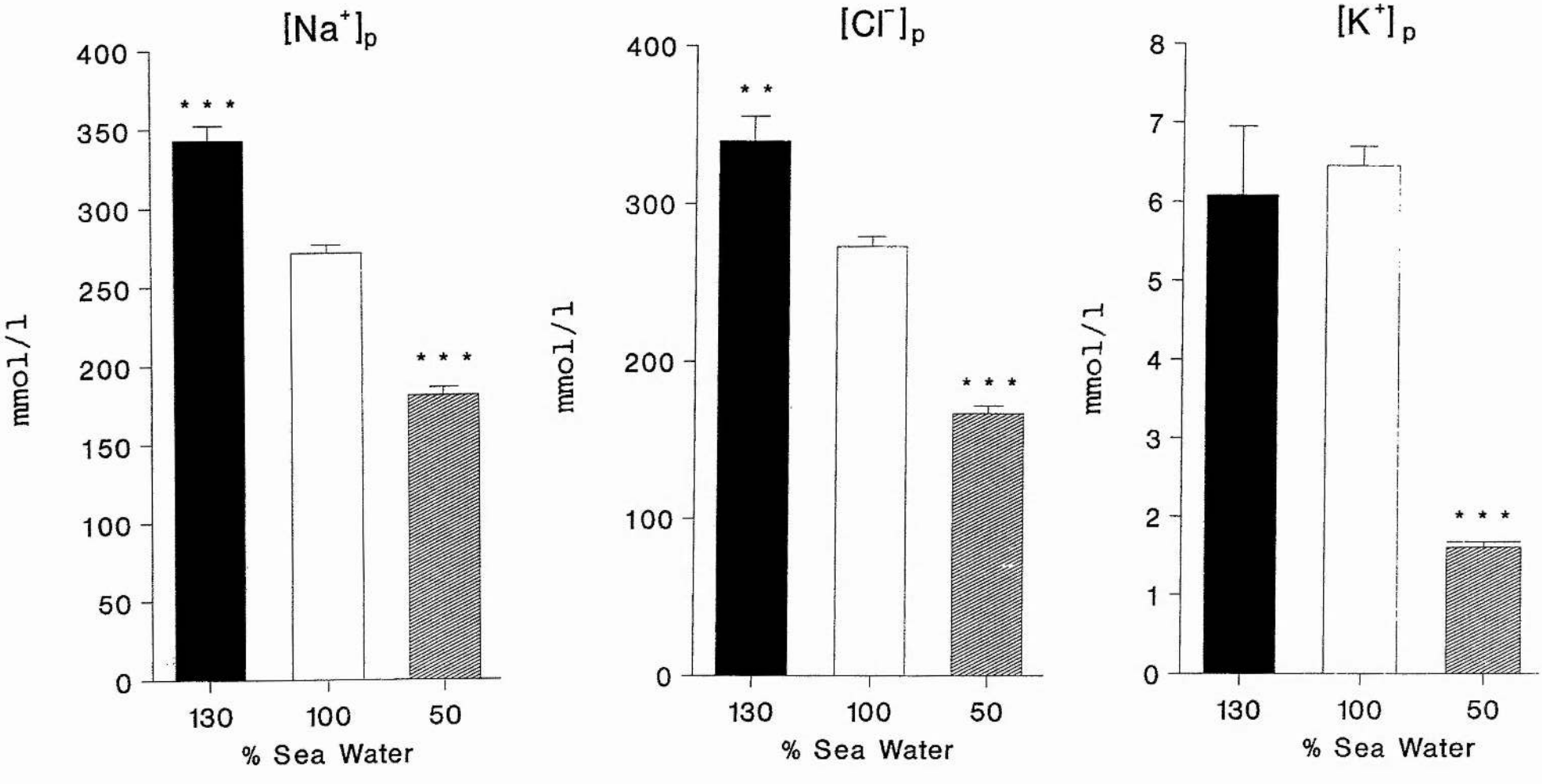
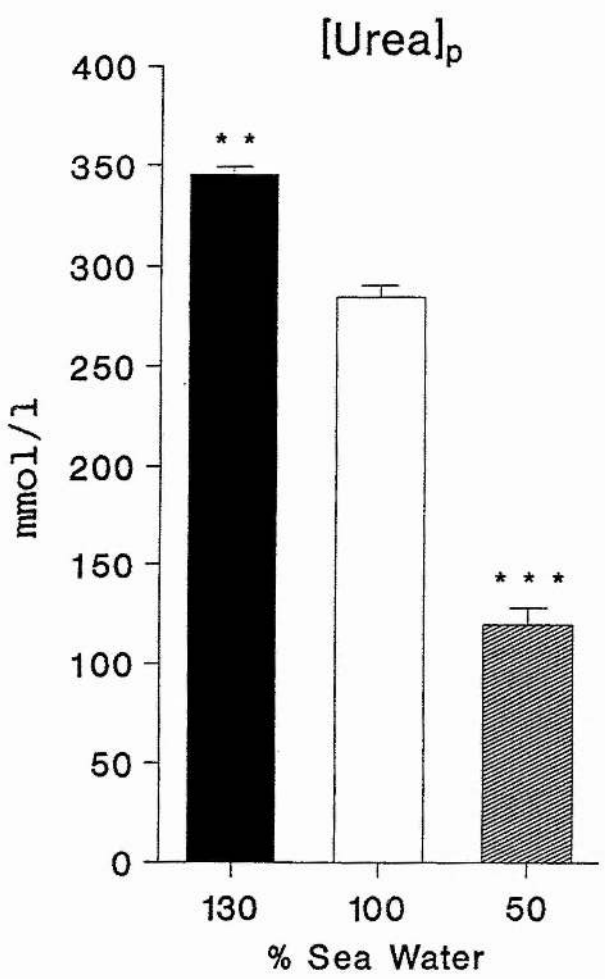
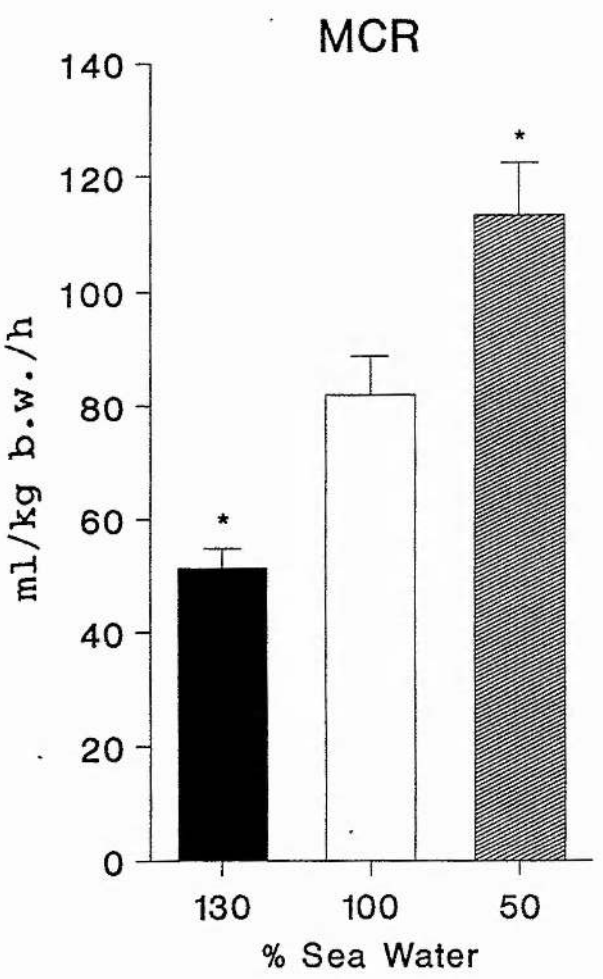
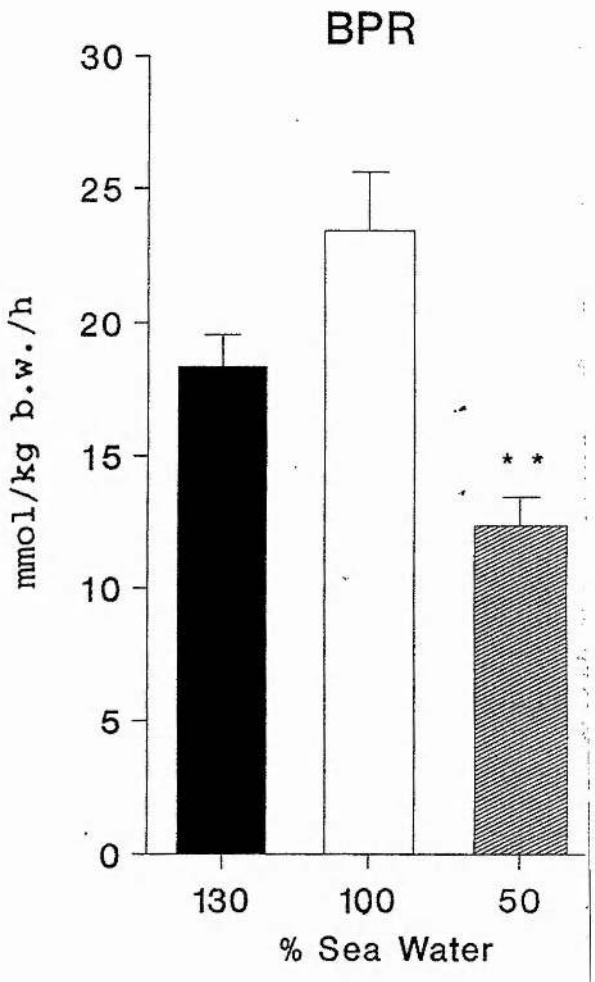

[Na+]p
400
350
300
250
200
150
100
50
0
mmol/l
* * *
* * *
130
100
50
% Sea Water
[Cl-]p
400
300
200
100
0
mmol/l
* *
* * *
130
100
50
% Sea Water
[K+]p
8
7
6
5
4
3
2
1
0
mmol/l
* * *
130
100
50
% Sea Water

Figure 4.7

Fig. 4.7 Urea Dynamics in High Protein Diet Fish
Urea dynamics in dogfish on a high protein diet adapted to normal seawater (clear bars), 130% seawater (solid bars) or 50% seawater (hatched bars). Plasma concentration ($[urea]_p$, in mmol/l), metabolic clearance rate (MCR, in ml/Kg body wt per hr.) and blood production rate (BPR, mmol/Kg body wt per hr.) are given. Results are means ± S.E.M. Number of animals = 7 in each case.
***, ** and * indicate statistically significant differences at $p<0.005$, $p<0.01$ and $p<0.05$, respectively (Student's t-test) from values seen in 100% seawater.


[Urea]p
mmol/l
400
350
300
250
200
150
100
50
0
* *
* * *
130
100
50
% Sea Water


MCR
ml/kg b.w./h
140
120
100
80
60
40
20
0
*
*
130
100
50
% Sea Water


BPR
mmol/kg b.w./h
30
25
20
15
10
5
0
* *
130
100
50
% Sea Water

concentration ($p<0.005$) was achieved by an increase in MCR ($p<0.05$) and a decrease in BPR ($p<0.01$).

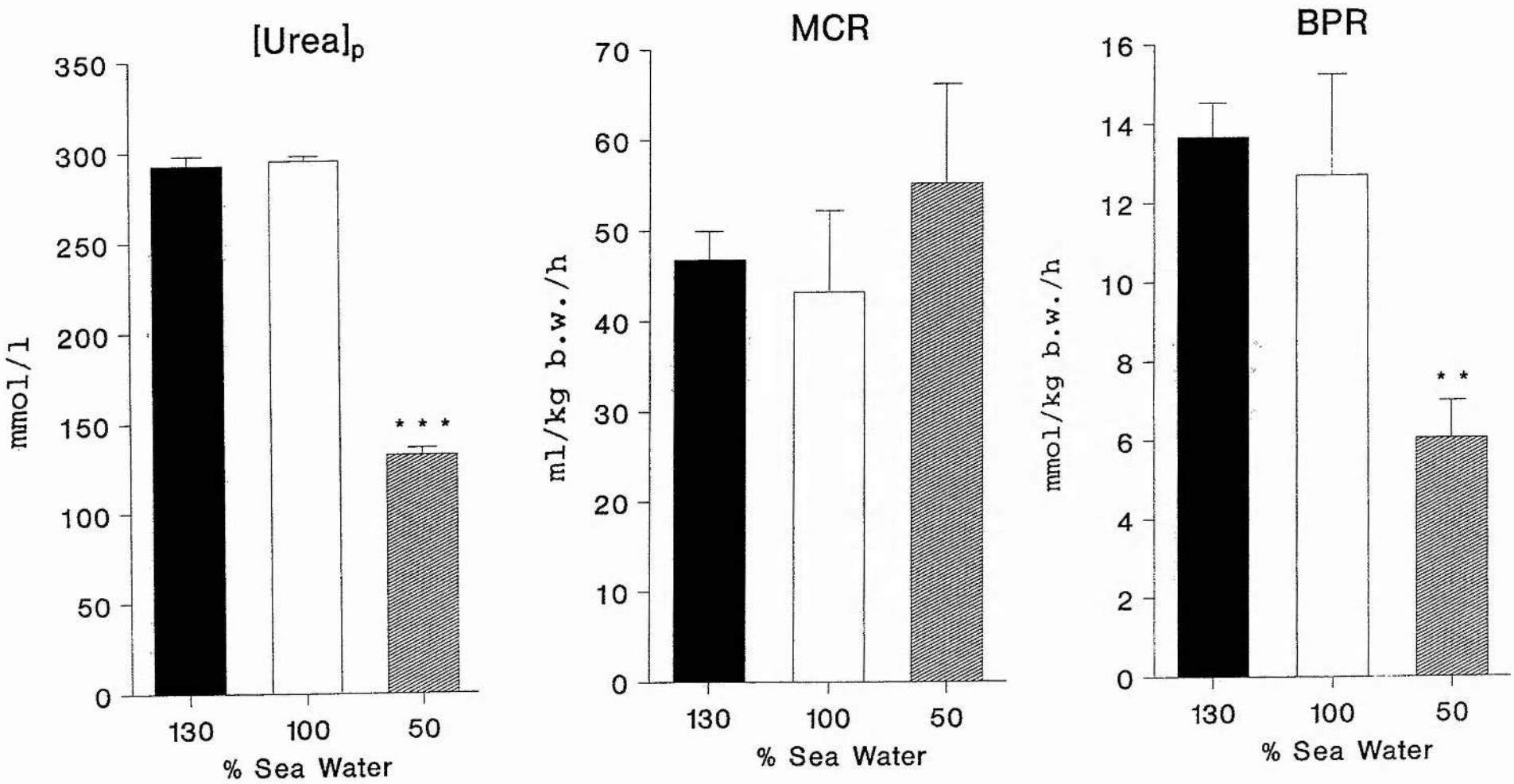
The urea dynamics of fish on a LPD are shown in Figure 4.8. There was no change in plasma urea concentration, MCR and BPR, in 130% seawater. The absence of a change in plasma urea concentration would explain the necessity for the very high plasma Na^+ levels measured in these fish, which were required to maintain an elevated iso-osmotic plasma in 130% seawater. In 50% seawater a reduction in plasma urea concentration ($p<0.005$) was observed and this was achieved by decreasing BPR ($p<0.01$). In normal and 50% seawater both dietary groups showed similar plasma urea levels but in 130% seawater LPD fish appeared unable to increase plasma urea concentration, in contrast to the HPD fish.

LPD fish showed no change in urea MCR in 130% seawater, presumably because clearance was already maximally reduced in 100% seawater and could not be decreased any further. In 130% seawater the HPD fish decreased their MCR to a level comparable with that observed in the LPD fish. The urea MCR's of LPD fish (and high protein fish in 130% seawater) are some of the lowest that have been measured in an elasmobranch and 40-50ml/Kg body weight per hour would appear to be at the physiological lower limit. BPR's of urea in LPD fish were significantly ($p<0.05$) lower than the corresponding values for HPD fish at the environmental osmolalities used.

Urea is the end product of protein (and nitrogen) metabolism in elasmobranchs and the lowered urea blood

Figure 4.8

Fig. 4.8 Urea Dynamics in Low Protein Diet Fish
Urea dynamics in dogfish on a low protein diet adapted to normal seawater (clear bars), 130% seawater (solid bars) and 50% seawater (hatched bars). Plasma concentration ($[urea]_p$, in mmol/l), metabolic clearance rate (MCR, in ml/Kg body wt per hr.) and blood production rate (BPR, mmol/Kg body wt per hr.) are given. Results are means ± S.E.M. Number of animals: for 130% seawater, n=8; for 100% seawater, n=11; and for 50% seawater, n=8.
*** and ** indicate statistically significant differences at $p<0.005$ and $p<0.01$ respectively (Student's t-test) from values seen in 100% seawater.


[Urea]p
mmol/l
350
300
250
200
150
100
50
0
* * *
130
100
50
% Sea Water
MCR
ml/kg b.w./h
70
60
50
40
30
20
10
0
130
100
50
% Sea Water
BPR
mmol/kg b.w./h
16
14
12
10
8
6
4
2
0
* *
130
100
50
% Sea Water

production rates in LPD fish reflect the restriction on protein intake imposed by such a LPD.

The results suggest that in normal seawater the LPD fish are, at best, just able to sustain urea production and the maintenance of plasma urea concentration. These parameters are vitally dependent on low metabolic clearance of urea. This of course provides no scope for adaptation to a hyperosmotic environment and LPD fish appear incapable of increasing urea BPR, and of further decreasing urea MCR, and consequently cannot increase their plasma urea concentration in 130% seawater.

Perhaps surprisingly, on adaptation to 50% seawater LPD fish, like HPD fish, decrease urea BPR but, unlike the HPD fish, they do not increase their metabolic clearance of urea. This suggests that while it may be energetically more favourable to decrease urea BPR than increase urea MCR under normal conditions, it may be especially so under conditions of severe stress such as combined long-term dietary protein restriction and chronic osmotic change.

4.4.5 1α-hydroxycorticosterone dynamics

Figure 4.9 shows the 1α-OH-B dynamics of HPD fish adapted to 130% and 50% seawater. Plasma 1α-OH-B concentration remained unchanged in 130% seawater but showed a significant increase in 50% seawater ($p<0.01$) and this was accompanied by an increase in 1α-OH-B BPR ($p<0.05$) and an increase in MCR ($p<0.05$).

Figure 4.10 shows the dynamics of 1α-OH-B in fish on a LPD. In 130% seawater there was a very large increase in

Figure 4.9

Fig. 4.9 1α-hydroxycorticosterone Dynamics in High Protein Diet Fish

Dynamics of 1α-hydroxycorticosterone (1α-OH-B) in dogfish on a high protein diet adapted to normal sewater (clear bars), 130% seawater (open bars) and 50% seawater (hatched bars). Plasma concentration ($[1\alpha\text{-OH-B}]_p$, in ng/ml), metabolic clearance rate (MCR, in ml/Kg body wt per hr.) and blood production rate (BPR, in ng/Kg body wt per hr.) are given. Results are means ± S.E.M. Numbers of animals are: for 130% seawater, n=7; for normal seawater, n=11; and for 50% seawater, n=7.

** and * indicate statistically significant differences at $p<0.01$ and $p<0.05$, respectively (Student's t-test) from values seen in 100% seawater.

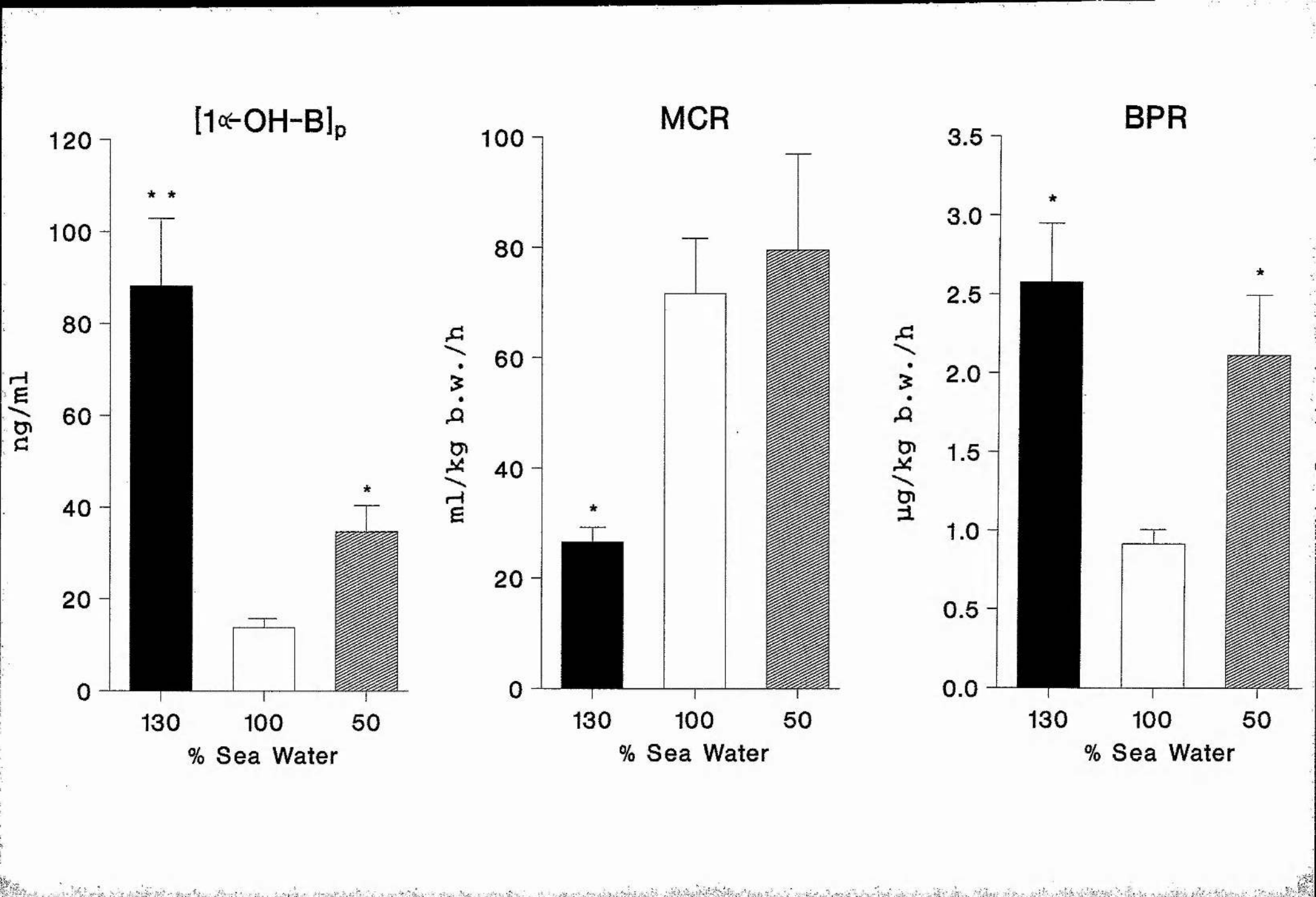
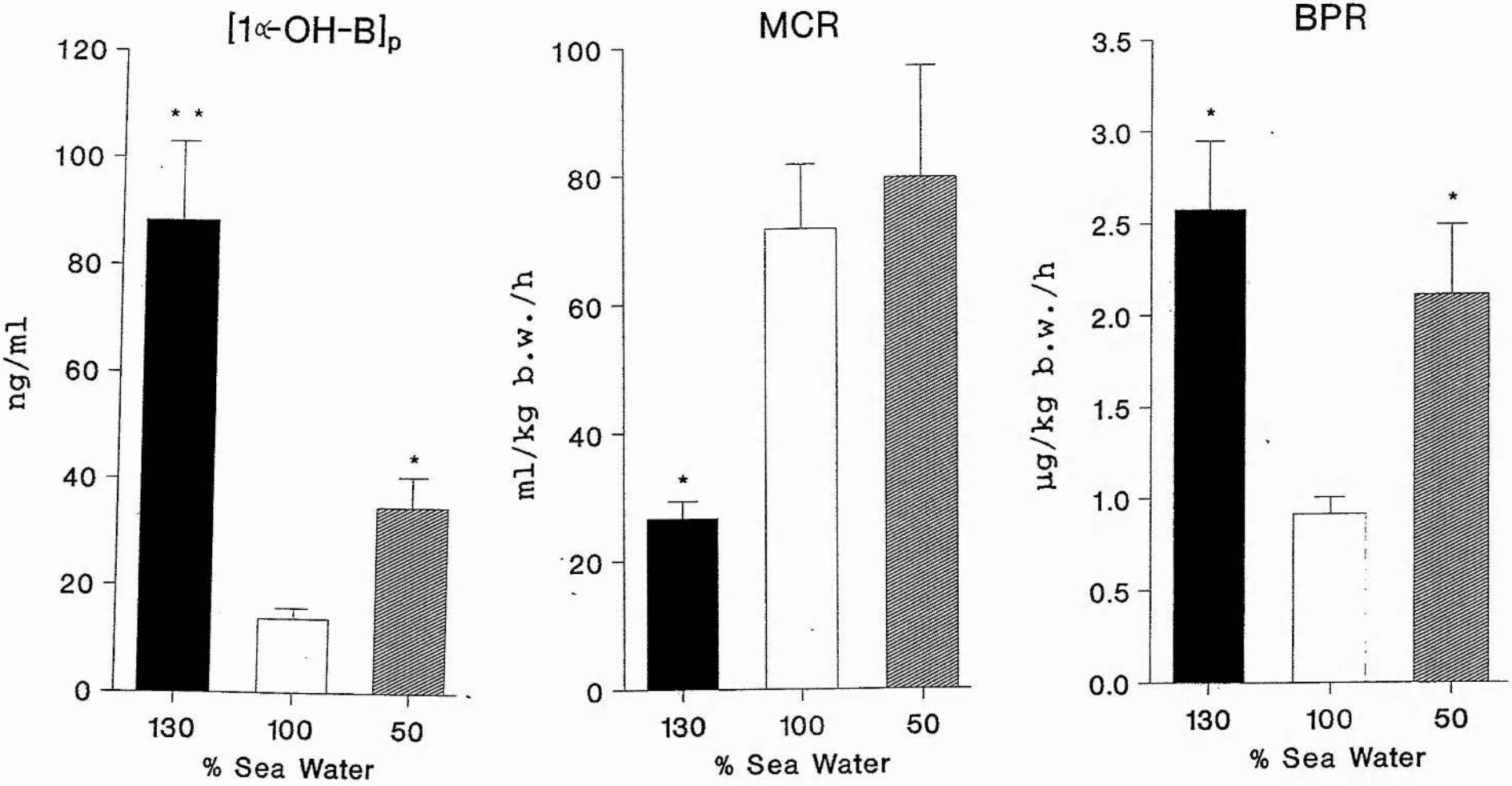

[1α-OH-B]p
ng/ml
120
100
80
60
40
20
0
* *
*
130
100
50
% Sea Water
MCR
ml/kg b.w./h
100
80
60
40
20
0
*
130
100
50
% Sea Water
BPR
μg/kg b.w./h
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
*
*
130
100
50
% Sea Water

Figure 4.10

Fig. 4.10 1α-hydroxycorticosterone Dynamics in Low Protein Diet Fish

Dynamics of 1α-hydroxycorticosterone (1α-OH-B) in dogfish on a low protein diet adapted to normal sewater (clear bars), 130% seawater (open bars) and 50% seawater (hatched bars). Plasma concentration ($[1\alpha\text{-OH-B}]_p$, in ng/ml), metabolic clearance rate (MCR, in ml/Kg body wt per hr.) and blood production rate (BPR, in ng/Kg body wt per hr.) are given. Results are means ± S.E.M. Numbers of animals are: for 130% seawater, n=6; for normal seawater, n=7; and for 50% seawater, n=6.

** and * indicate statistically significant differences at $p<0.01$ and $p<0.05$, respectively (Student's t-test) from values seen in 100% seawater.


[1α-OH-B]p
ng/ml
120
100
80
60
40
20
0
* *
*
130
100
50
% Sea Water
MCR
ml/kg b.w./h
100
80
60
40
20
0
*
130
100
50
% Sea Water
BPR
µg/kg b.w./h
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
*
*
130
100
50
% Sea Water

plasma 1α-OH-B concentration ($p<0.01$) and this was achieved by decreased MCR ($p<0.05$) and increased BPR ($p<0.05$). In 50% seawater there was a large increase in plasma 1α-OH-B concentration ($p<0.05$) and this was achieved by an increase in BPR ($p<0.05$).

The high plasma 1α-OH-B concentrations in 130% and 50% seawater correspond to periods of Na^+ regulation in these animals and the results suggest that 1α-OH-B may have a mineralocorticoid role, regulating plasma Na^+ and electrolyte concentrations. The 1α-OH-B concentrations in 130% seawater are the highest that have been measured and may reflect the very high degree of compensation which is required for survival under the combined stresses of dietary protein restriction and high osmotic environment.

In 100% seawater the plasma concentrations of 1α-OH-B were similar in both dietary groups, as were the MCR's and BPR's.

In 50% seawater both dietary groups exhibited significantly increased plasma 1α-OH-B concentrations but those measured for the HPD fish were significantly greater ($p<0.05$) than those for the LPD fish. Both the 1α-OH-B MCR and BPR were also suppressed in LPD fish compared to the values obtained for fish on a HPD.

The comparatively lower plasma concentration of 1α-OH-B in LPD fish would be entirely appropriate given a role for 1α-OH-B in sodium regulation. It is possible that LPD fish in 50% seawater, which exhibit a lower clearance rate of urea than HPD fish, may also have lower electrolyte

clearance than HPD fish and therefore, as a consequence, require a smaller increase in plasma 1α-OH-B to sustain controlled osmoregulation.

The absence of increased plasma 1α-OH-B concentration in HPD fish in 130% seawater compared to the very large increase observed in LPD fish is physiologically quite appropriate. Without the constraints imposed by dietary protein restriction, fish on a HPD are capable of increasing their plasma urea concentration proportionately with the increasing environmental osmolality. As a result, while plasma sodium concentration also rises, there is no necessity for the extreme increase in plasma sodium concentration observed, perhaps as a result of drinking, in LPD fish. Consequently, an increase in plasma 1α-OH-B concentration to regulate sodium and electrolyte balance during high osmotic stress is not observed.

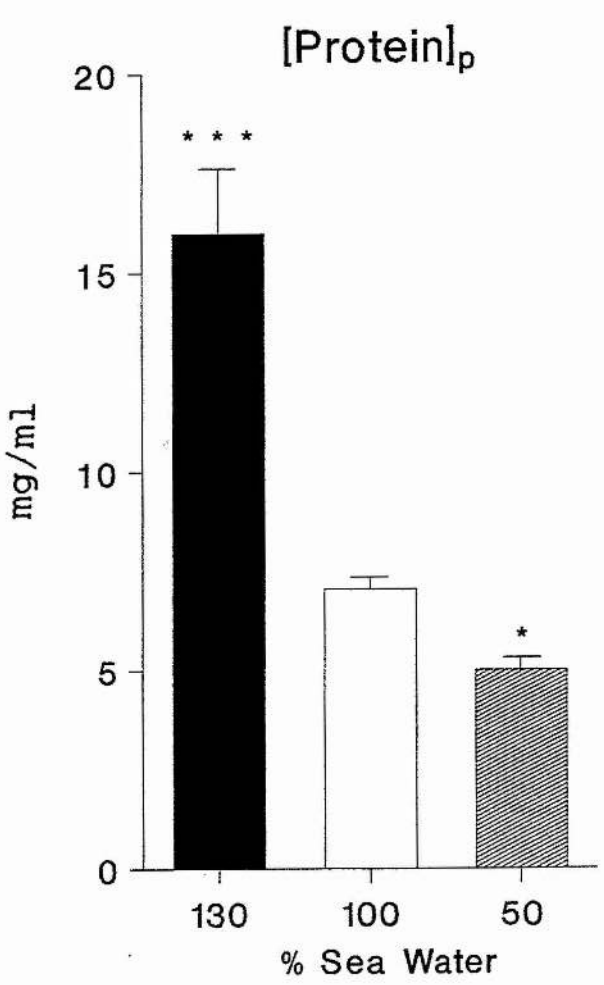
4.4.6 Changes in plasma protein, lipid and cholesterol concentrations

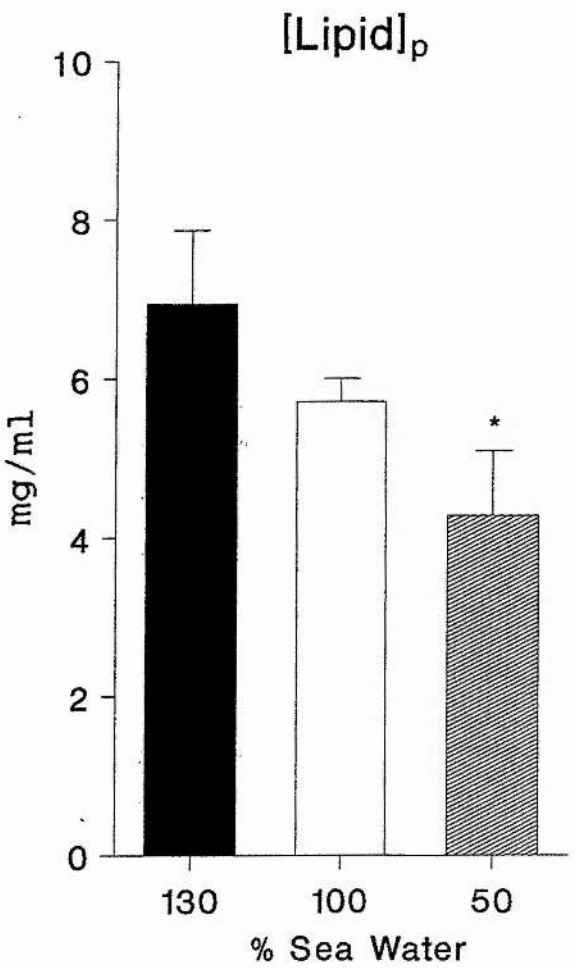
The changes in plasma protein, lipid and cholesterol concentrations were similar in both high (Figure 4.11) and low (Figure 4.12) protein dietary groups and varied in proportion to changes in environmental osmolality. In 50% seawater plasma protein, lipid and cholesterol concentrations were all significantly reduced. In 130% seawater their plasma concentrations were all increased, although the increases in lipid concentration were not statistically significant. The increases observed in 130% seawater were possibly the result of slightly decreased

Figure 4.11

Fig. 4.11 Changes in Plasma Protein, Lipid and Cholesterol Concentrations in High Protein Diet Fish

Plasma concentrations of protein (mg/ml), lipid (mg/ml) and cholesterol (mmol/l) in dogfish on a high protein diet adapted to 100% (clear bars), 130% (solid bars) and 50% seawater (hatched bars). Results are means ± S.E.M. Numbers of animals: for 130% seawater, n=5; for 100% seawater, n=10; and for 50%, n=11.
***, ** and * indicate statistically significant differences at $p<0.005$, $p<0.01$ and $p<0.05$, respectively (Student's t-test) compared with values seen in 100% seawater.


[Protein]p
mg/ml
20
15
10
5
0
* * *
*
130
100
50
% Sea Water


[Lipid]p
mg/ml
10
8
6
4
2
0
*
130
100
50
% Sea Water

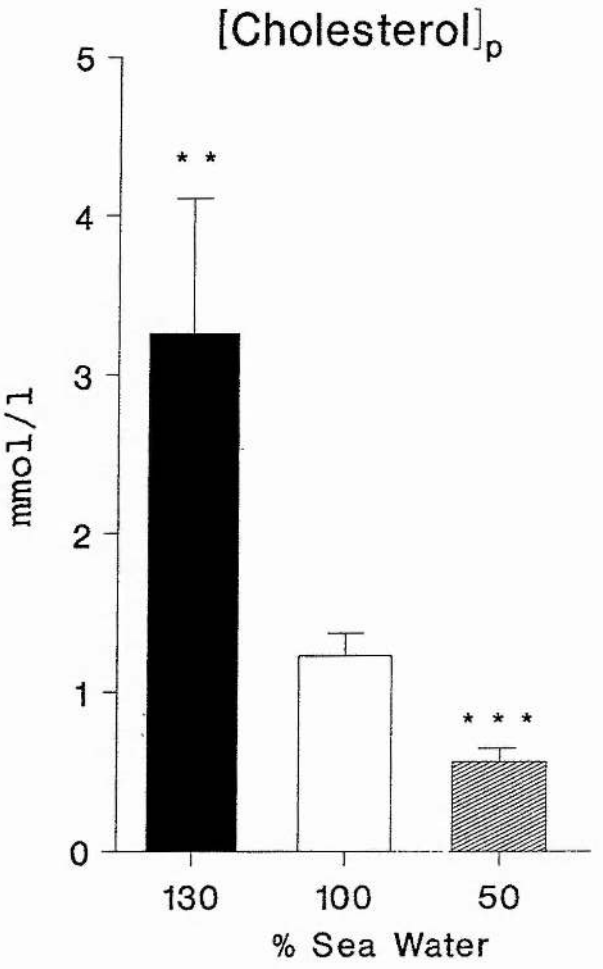
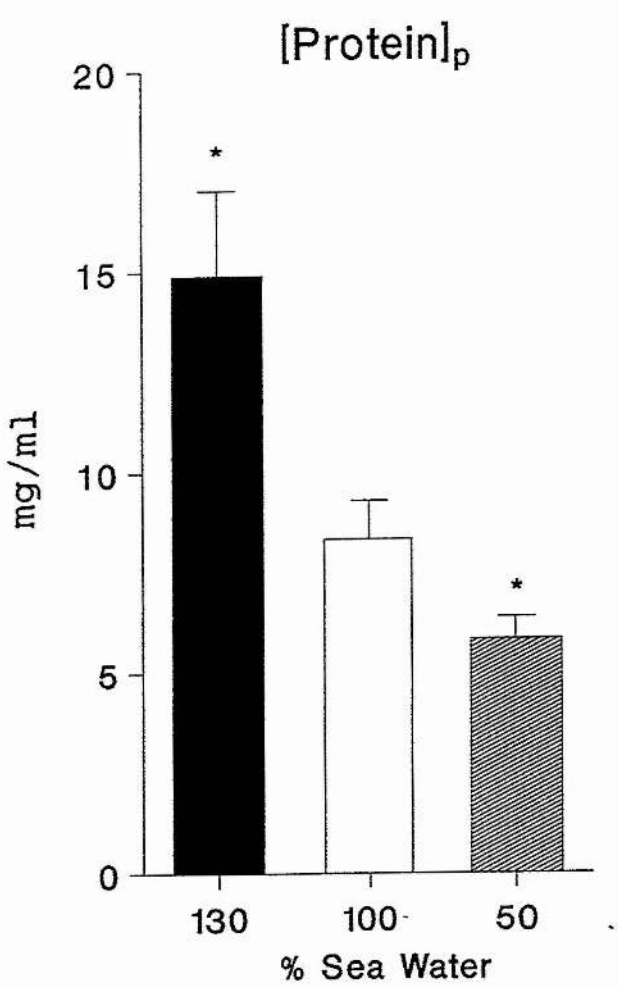
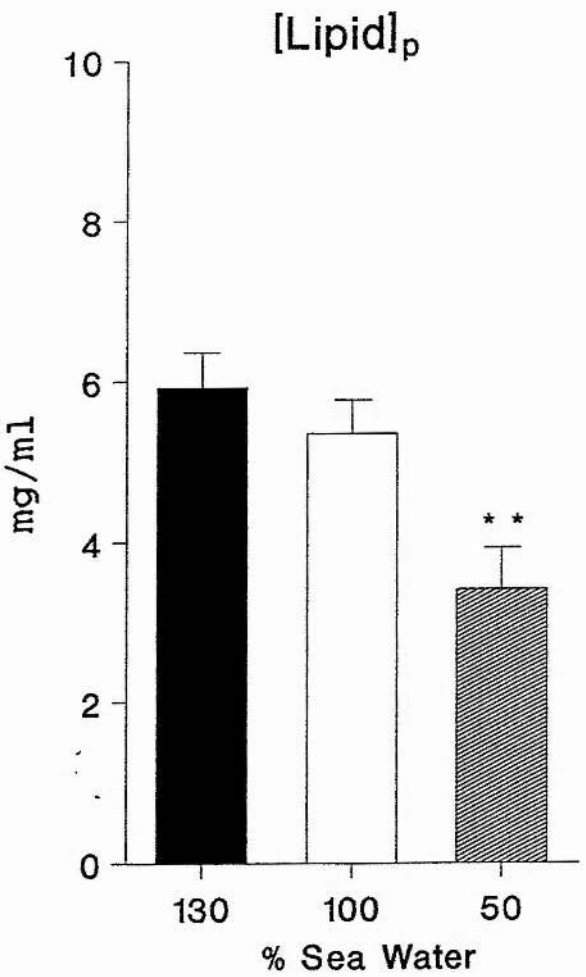

[Cholesterol]p
mmol/l
5
4
3
2
1
0
* *
* * *
130
100
50
% Sea Water

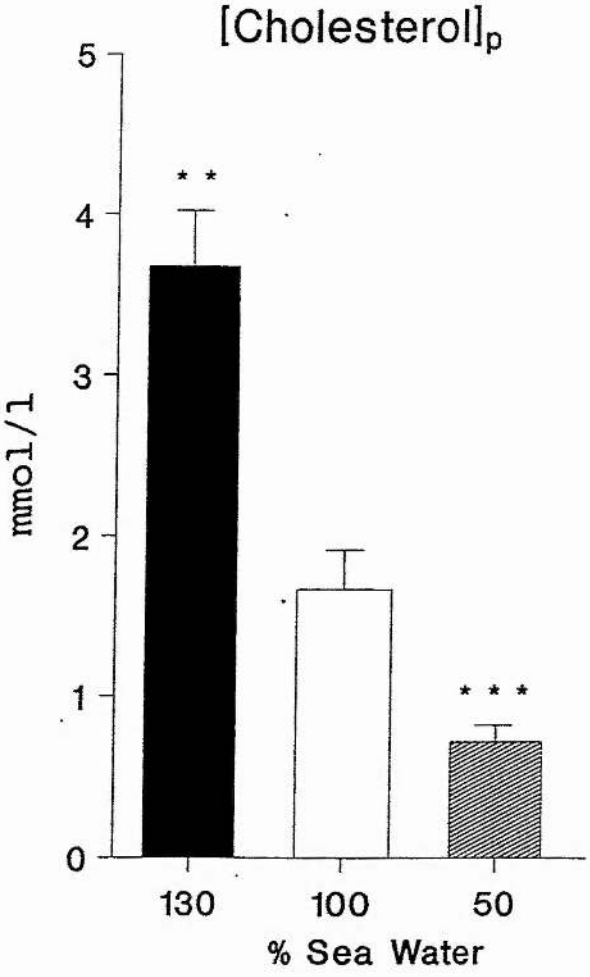
Figure 4.12

Fig. 4.12 Changes in Plasma Protein, Lipid and Cholesterol Concentrations in Low Protein Diet Fish

Plasma concentrations of protein (mg/ml), lipid (mg/ml) and cholesterol (mmol/l) in dogfish on a low protein diet adapted to 100% (clear bars), 130% (solid bars) and 50% seawater (hatched bars). Results are means ± S.E.M. Numbers of animals: for 130% seawater, n=5; for 100% seawater, n=10; and for 50%, n=5.
***, ** and * indicate statistically significant differences at $p<0.005$, $p<0.01$ and $p<0.05$, respectively (Student's t-test) compared with values seen in 100% seawater.


[Protein]p
mg/ml
20
15
10
5
0
*
*
130
100
50
% Sea Water


[Lipid]p
mg/ml
10
8
6
4
2
0
* *
130
100
50
% Sea Water


[Cholesterol]p
mmol/l
5
4
3
2
1
0
* *
* * *
130
100
50
% Sea Water

plasma volume, a common consequence of experimental adaptation to a hyperosmotic environment, and a general decrease in clearance, in the case of HPD fish, which show slightly greater increases in plasma protein, lipid and cholesterol concentration than do the LPD fish. In 50% seawater the decreases observed may be a result of slight increases in plasma volume, combined with increased clearance in HPD fish.

Plasma protein levels did not significantly differ between the two dietary groups. This is perhaps not surprising as the protein used for urea synthesis is found in its metabolized form in the plasma, as ammonia and amino acids, and is not detected by the Bradford assay.

Plasma lipid concentrations did not differ between the two dietary groups. This interesting observation indicates that the considerably higher content of lipid in the LPD does not produce an elevation in plasma total lipid concentration, compared to the HPD. This may reflect differences in gut absorption of high levels of lipid, or possibly indicates that the excess lipid in the LPD is being absorbed and stored in the liver, as it is well known that elasmobranchs retain high hepatic lipid concentrations (in chronically starved Scyliorhinus canicula liver lipid content of 45 ± 2% of total liver weight (n=10) has been measured (Armour and Hazon 1989, unpublished observation) and significantly higher values would be expected in fed animals).

The plasma cholesterol results are also of significant interest. Plasma cholesterol concentration, which does not

differ between the two dietary groups, seems to mirror changes in plasma osmolality and not changes in plasma corticosteroid concentration. These results indicate that the diets used in this study (especially the LPD), although providing a ready source of cholesterol, an essential precursor of steroid biosynthesis, are not directly stimulating steroid biosynthesis.

4.5 In vitro determination of hepatic urea production

Table 4.4 compares the values obtained for in vitro urea production in this study with those published values in the literature. In the preliminary studies, without doubt, the most accurate and, at the time, most reproducible technique involved preparation of hepatocytes (see Table 4.4). However, during the osmotic adaptation studies, a major problem emerged that was not present during the development of this technique. This was seasonal variability in producing isolated hepatocytes that apparently depended upon liver lipid content. In effect this meant that it was impossible to produce predictable isolated cell preparations from the experimental groups. The technique was based upon modifications of the method described by Mommsen and Moon (1987). It was later confirmed that a seasonal variation was also a major (unsolved) problem with the skate hepatocyte preparation (Moon, 1989, Personal Communication).

Table 4.4

Table 4.4 Comparison of Urea Production in Different In Vitro Preparations

Table 4.4 Comparison of Urea Production in Different In Vitro Preparations

	Urea Production (µmol/g/hr)		
Species	Tissue Slices	Homogenates	Hepatocytes
Scyliorhinus canicula	0.28 ± 0.11	1.73 ± 0.53	1.09 ± 0.19
Squalus acanthias	2.02 ± 2.54 (Schooler et al, 1966)	-	-
Raja erinacea	-	-	0.85 ± 0.20 (Mommsen & Moon, 1987)

4.6 Experiments on isolated hepatic mitochondria

4.6.1 Assay of carbamoyl synthetase III activity

Using the method of Ritter, Smith and Campbell (1987), no significant CPS III activity was detected in isolated mitochondria from any region of the dogfish liver. Neither was CPS III activity detected in any of the subcellular fractions obtained during mitochondrial isolation.

This was examined further by testing the effects of the procedural variations listed in Fig. 3.2.

(i) None of the variations tested on the preparation of hepatic subcellular fractions resulted in detectable CPS III activity.

(ii) Varying the amount of mitochondrial protein added and varying the specific activity of the $[^{14}C]HCO_3$ used, presented no significant improvement.

(iii) The use of reaction mixtures resembling either elasmobranch intracellular of extracellular fluids (Table 3.2) also did not result in detectable CPS III activity.

It was concluded that either the assay procedure or the mitochondrial preparation used was at fault and initially another assay for the stoichiometric measurement of urea synthesis was tested.

4.6.2 Assay of citrulline synthesis

The assay employed by Anderson and Casey (1984) and Anderson (1986) to measure the rate of citrulline synthesis proved ineffectual on isolated mitochondrial preparations

from Scyliorhinus canicula and no significant amounts of citrulline were detected.

To examine this further the procedural variations listed in Fig 3.2 were carried out. Neither the variations in the mitochondrial isolation procedure nor the alterations in assay procedure, which included the use of reaction mixtures resembling elasmobranch intracellular and extracellular fluids (Table 3.3), resulted in detectable amounts of citrulline synthesis.

The results of the assay procedures tried suggested that the hepatic mitochondrial preparations used might not be viable and the obvious next step was to test hepatic mitochondrial respiration.

4.6.3 Mitochondrial respiration

Following the procedure of Anderson (1986), the state 3 rate of hepatic mitochondrial respiration was found to be 2.36 ± 0.13 nmol O_2/min per mg and the respiratory control ratio was found to be 1.2 ± 0.1. The measured state 3 respiration rate is 4-6 fold less than the quoted range of 10-15nmol O_2/min per mg (Anderson, 1986) and the respiratory control ratio, a measure of mitochondrial viability, is less than half the value regarded as the minimum acceptable ratio for a viable mitochondrial preparation (Anderson, 1986). The addition of the uncoupling agent dinitrophenol (DNP 0.1mM) had no apparent effect on the rate of respiration, further indicating that the isolated mitochondrial preparations used were not viable.

Mitochondrial respiration was examined further using respiration buffers based on elasmobranch intracellular and extracellular fluids. The intracellular fluid-based buffer represented no significant improvement over the buffer originally used and the extracellular fluid-based buffer actually produced decreased mitochondrial respiration (to 1.14 ± 0.19nmol/mg/min). Using the mitochondrial isolation procedure followed by Ritter, Smith and Campbell (1987) similar results to those presented above were obtained.

The first two enzymes in the elasmobranch urea cycle, glutamine synthetase and CPS III are believed to be the rate controlling enzymes for urea synthesis (Anderson, 1981, 1986; Shankar and Anderson, 1985). In the current study the measurements of carbamoyl phosphate and citrulline formation (and hence urea synthesis) were undertaken in an attempt to assess the effect of dietary protein restriction on the activities of these two enzymes. The procedures used in the current study were developed for use in Hydrolagus collei (Ritter, Smith and Campbell, 1987) and Squalus acanthias (Anderson and Casey, 1984) and their failure to work in Scyliorhinus canicula may be attributable to the different species used. In particular the poor viability of the isolated mitochondria from Scyliorhinus canicula may indicate species differences in the ultrastructure of liver tissue.

4.7 Studies on the isolated perifused interrenal gland

4.7.1 Validation of the preparation

During the initial 2 hours of perifusion there was a clear wash-out of steroid as illustrated by Figure 4.13a. Continued perifusion demonstrated that the tissue remained viable for long periods and was still responsive to a dose of 10μM dibutyryl (Bt_2) cAMP after 21 hours (Figure 4.13b).

Following the 2 hour wash-out period and during the control hour of each dose-response cycle a steady-state basal secretion rate was established. The steady state basal secretion rates for each experiment are quoted in the relevant figure legends which follow and, unless otherwise stated, all values quoted are means ± S.E.M.

4.7.2 Effect of increasing doses of porcine ACTH

Porcine ACTH (0.1 - 10μM) produced a dose-dependent increase in 1α-OH-B production (Figure 4.14), the maximum stimulation being 280 ± 38% (S.E.M.) above basal secretory rate ($p<0.01$). Heterologous ACTH was used and it is reasonable to assume that homologous ACTH would be a more potent stimulant of steroidogenesis in the dogfish interrenal gland. The response to porcine ACTH was immediate and the peak occurred within 15-30 minutes of stimulation. The duration of the response increased with dose and ranged from $1^3/_4$ - $2^1/_4$ hours following a maximal dose of 10μM porcine ACTH.

4.7.3 Effect of dibutyryl cyclic AMP

Dibutyryl cAMP (10pM - 10μM) produced a dose-dependent increase in 1α-OH-B production (Figure 4.15), with a

Figure 4.13

Fig. 4.13 Mean Secretory Rates of 1α-hydroxycorticosterone from two dogfish interrenal glands perifused over a 22-hour period
a) Initial wash-out of steroid for 2 hours before attaining a steady basal secretory rate and
b) The effect of 10μM dibutyryl cyclic AMP on 1α-OH-B after 21 hours.

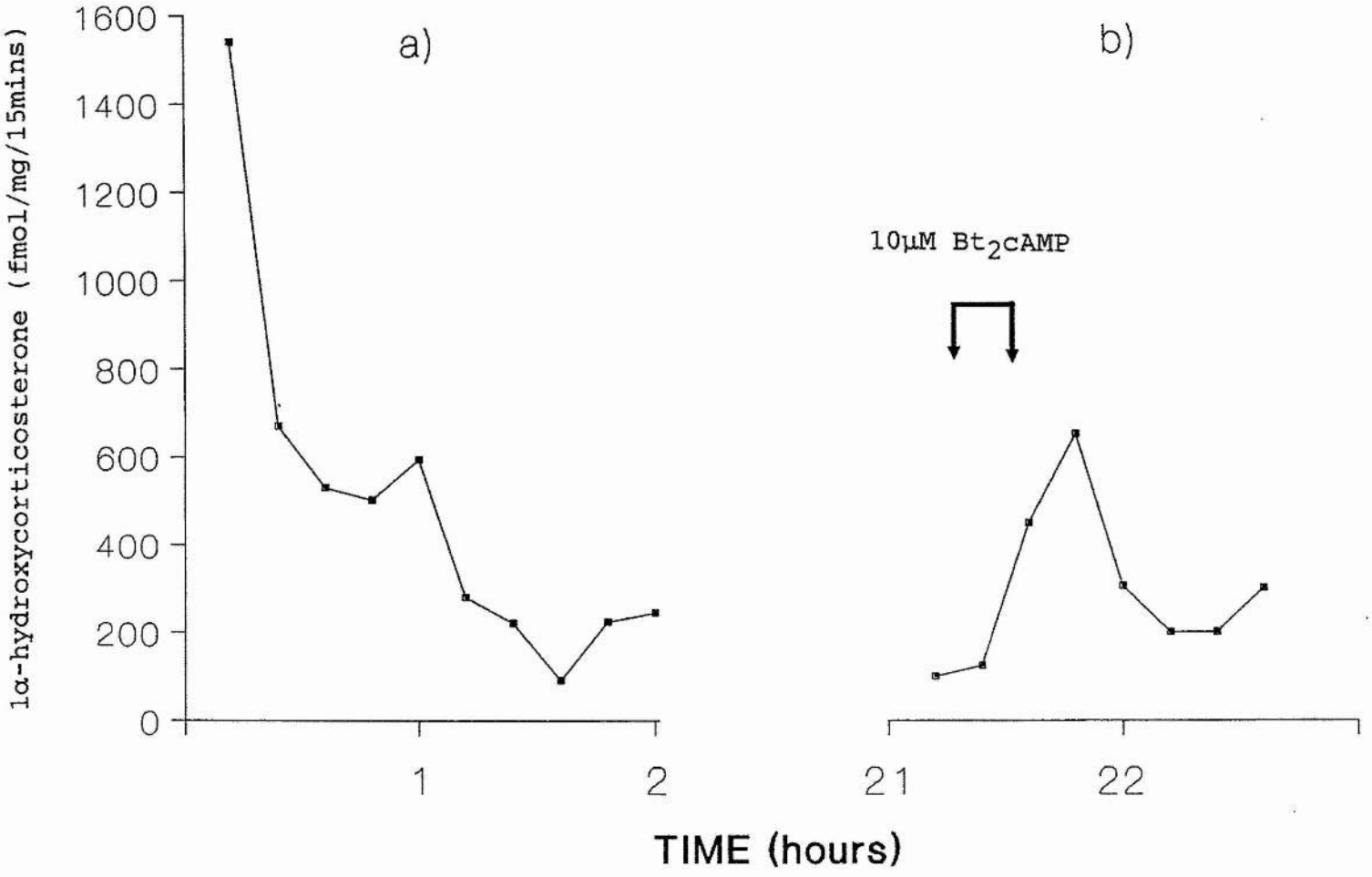

1α-hydroxycorticosterone (fmol/mg/15mins)
1600
1400
1200
1000
800
600
400
200
0
a)
b)
10μM Bt2cAMP
1
2
21
22
TIME (hours)

Figure 4.14

Fig. 4.14 Effect of increasing doses of porcine ACTH on 1α-hydroxycorticosterone production
Each interrenal gland was allowed 2 hours to recover between each challenge. Results are means ± S.E.M. (n=5 or 6 as indicated below) of 1α-OH-B production expressed as percentage increases above basal secretory rates. Mean basal secretory rates were:
(i) for 0.01μM ACTH, 6236 ± 436, 5157 ± 545, 6174 ± 545, 6236 ± 352 and 2703 ± 198 fmol/mg per 15 min;
(ii) for 0.1μM ACTH, 4313 ± 506, 6404 ± 516, 3692 ± 144, 5165 ± 568 and 2160 ± 231 fmol/mg per 15 min;
(iii) for 1.0μM ACTH, 5701 ± 469, 4631 ± 684, 5168 ± 830, 5856 ± 544, 449 ± 21 and 2560 ± 415 fmol/mg per 15 min;
(iv) for 10μM ACTH, 5490 ± 650, 4862 ± 875, 4862 ± 840, 321 ± 73 and 1988 ± 212 fmol/mg per 15 min.
* $p<0.05$, ** $p<0.01$ compared with basal secretory rate (Student's t-test).

NB With hindsight, it would have been better to have analysed all of the perifusion data using a paired t-test since the Student's t-test, while giving qualitatively similar results to the paired t-test, slightly under-estimates the levels of significance when applied to related groups of data.

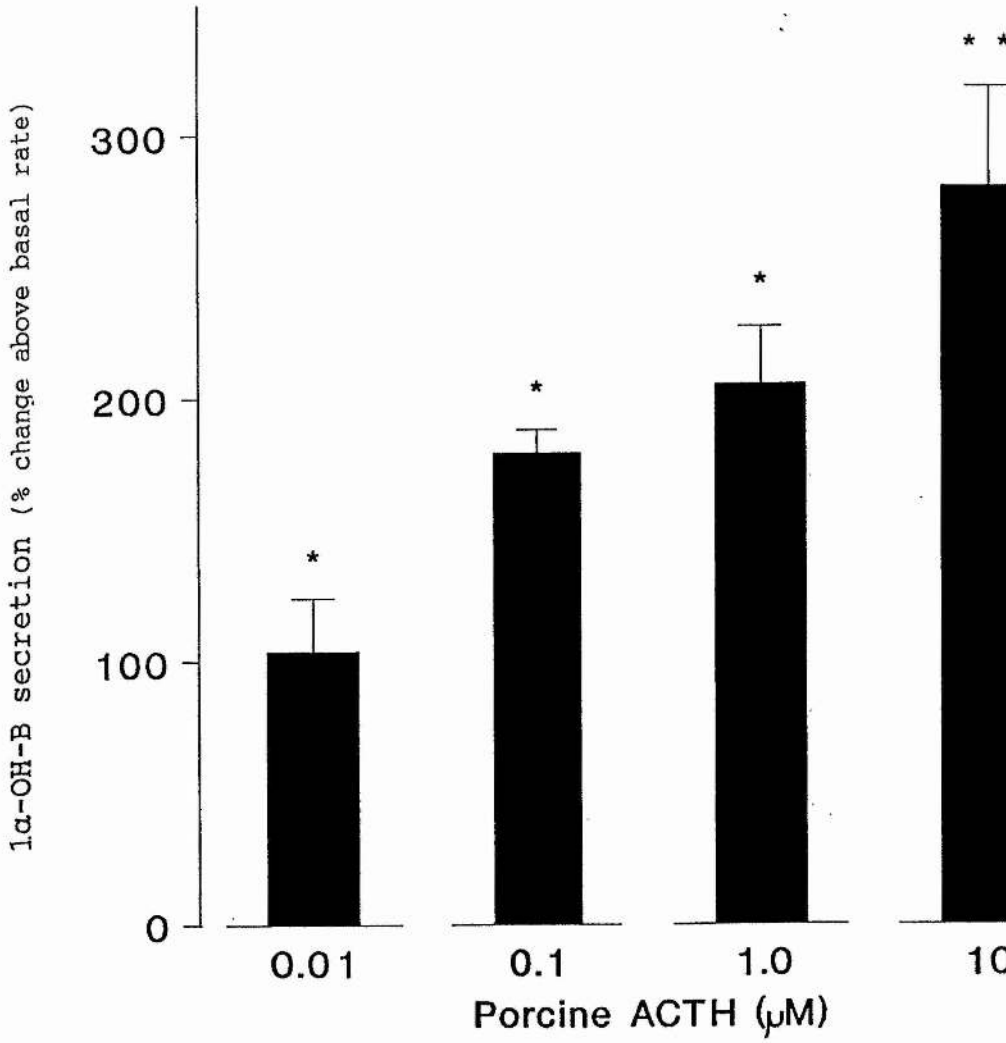
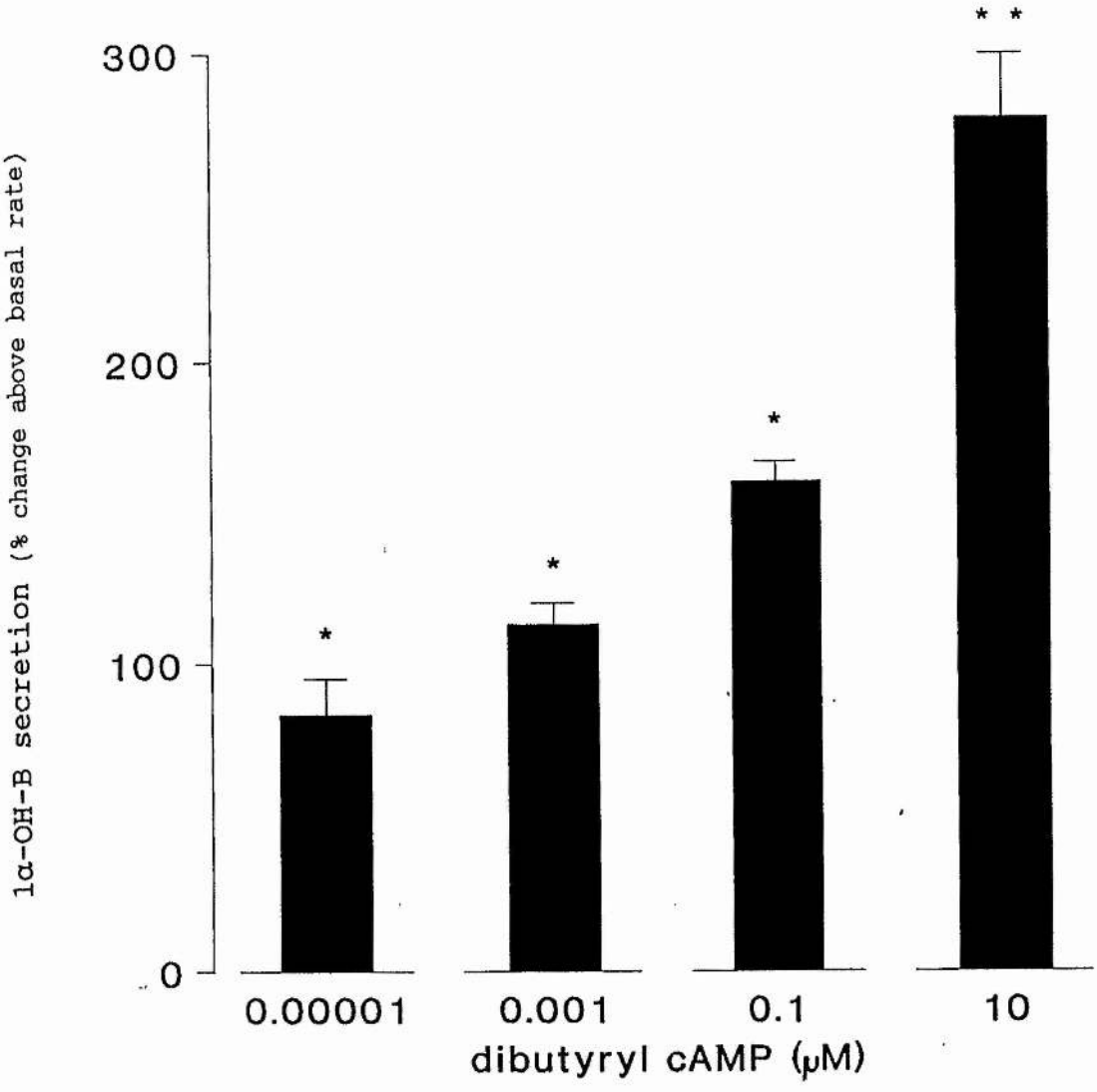

1α-OH-B secretion (% change above basal rate)
300
200
100
0
0.01
0.1
1.0
Porcine ACTH (μM)

Figure 4.15

Fig. 4.15 Effect of Increasing Doses of Dibutyryl Cyclic AMP on 1α-hydroxycorticosterone production
Each interrenal gland was allowed 2h recovery between each challenge. Results are means ± S.E.M. of 1α-OH-B production expressed as percentage increases above basal secretory rate. Mean basal secretory rates were:
(i) for 0.00001μM dibutyryl cAMP (n=3) 3408 ± 523, 4594 ± 782 and 5337 ± 971 fmol/mg per 15 min;
(ii) for 0.001μM dibutyryl cAMP (n=3) 3570 ± 185, 4412 ± 343 and 1250 ± 109 fmol/mg per 15 min;
(iii) for 0.01μM dibutyryl cAMP 674 ± 49, 8090 ± 545, 3743 ± 411 and 2182 ± 272 fmol/mg per 15 min;
(iv) for 0.1μM dibutyryl cAMP (n=14) 877 ± 145 fmol/mg per 15 min.
* $p<0.05$, ** $p<0.01$ compared with basal secretory rate (Student's t-test).


1α-OH-B secretion (% change above basal rate)
300
200
100
0
* *
*
*
*
0.00001
0.001
0.1
10
dibutyryl cAMP (μM)

maximal response of 278 ± 19% (S.E.M.) above basal secretory rate ($p<0.01$). Response to dibutyryl cAMP was immediate, peaking within 15-30 minutes after administration. The duration of response increased with dose and ranged from 1-2 hours following a dose of 10μM Bt_2cAMP.

These results demonstrate the existence of a cAMP-dependent cascade system in dogfish interrenal cells, which leads to stimulation of 1α-OH-B production.

4.7.4 Comparative effects of dibutyryl cAMP and forskolin

A standard dose of 10μM Bt_2cAMP produced a significant increase ($p<0.01$) in 1α-OH-B production (Figure 4.16) to 278 ± 19% (S.E.M.) above the basal secretory rate as indicated above. 1μM forskolin also produced a significant increase ($p<0.05$) in 1α-OH-B production to 130 ± 30% (S.E.M.) above basal secretory rate (Figure 4.16). The peak response to forskolin stimulation occurred within 30 minutes and the duration of response was typically 1½-2¼ hours. Forskolin is a direct stimulant of adenylate cyclase, which produces cAMP. Taken together the responses to Bt_2cAMP and forskolin demonstrate the existence of an endogenous adenylate cyclase/cAMP dependent cascade system which is involved in the stimulation of 1α-OH-B production in dogfish interrenal cells.

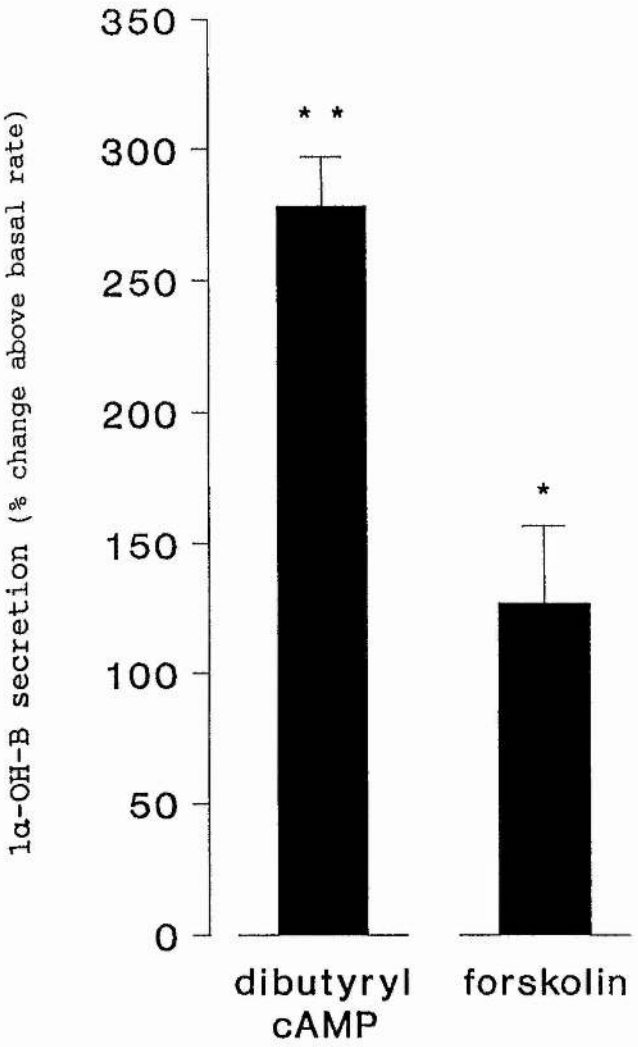
4.7.5 Effect of cholera toxin

The presence of cholera toxin in the perifusion medium for 3 hours at doses of 2μg/ml and 4μg/ml had no

Figure 4.16

Fig. 4.16 Comparison of the Effects of Dibutyryl cAMP and Forskolin

Effects of 10μM dibutyryl cyclic AMP (cAMP, n=14) and 1μM forskolin (n=4) on 1α-hydroxycorticosterone (1α-OH-B) production by perifused dogfish interrenal glands. Results are means ± S.E.M. of 1α-OH-B production expressed as percentage increases over basal secretory rates. Mean basal secretory rates were:
(i) for 10μM dibutyryl cAMP 877 ± 145 fmol/mg per 15 mins (mean ± S.E.M., n=14);
(ii) for 1μM forskolin 441 ± 49, 1364 ± 179 and 2210 ± 612 fmol/mg per 15 mins for individual glands.
* $p<0.05$, ** $p< 0.01$ compared with basal secretory rate (Student's t-test).


1α-OH-B secretion (% change above basal rate)
350
300
250
200
150
100
50
0
* *
*
dibutyryl cAMP
forskolin

significant effect on the basal secretory rate of 1α-OH-B (n=3 in each case).

Cholera toxin is an highly specific agent which catalyses the ADP ribosylation of stimulatory G proteins resulting in persistent activation of adenylate cyclase. The fact that cholera toxin did not affect basal secretion of 1α-OH-B may suggest that G proteins are not involved in the mediation of basal secretion in vitro. In vivo this is hardly likely to be the case since the interrenal gland will be exposed to a variety of circulating agonists and antagonists many of which will operate via G protein mechanisms.

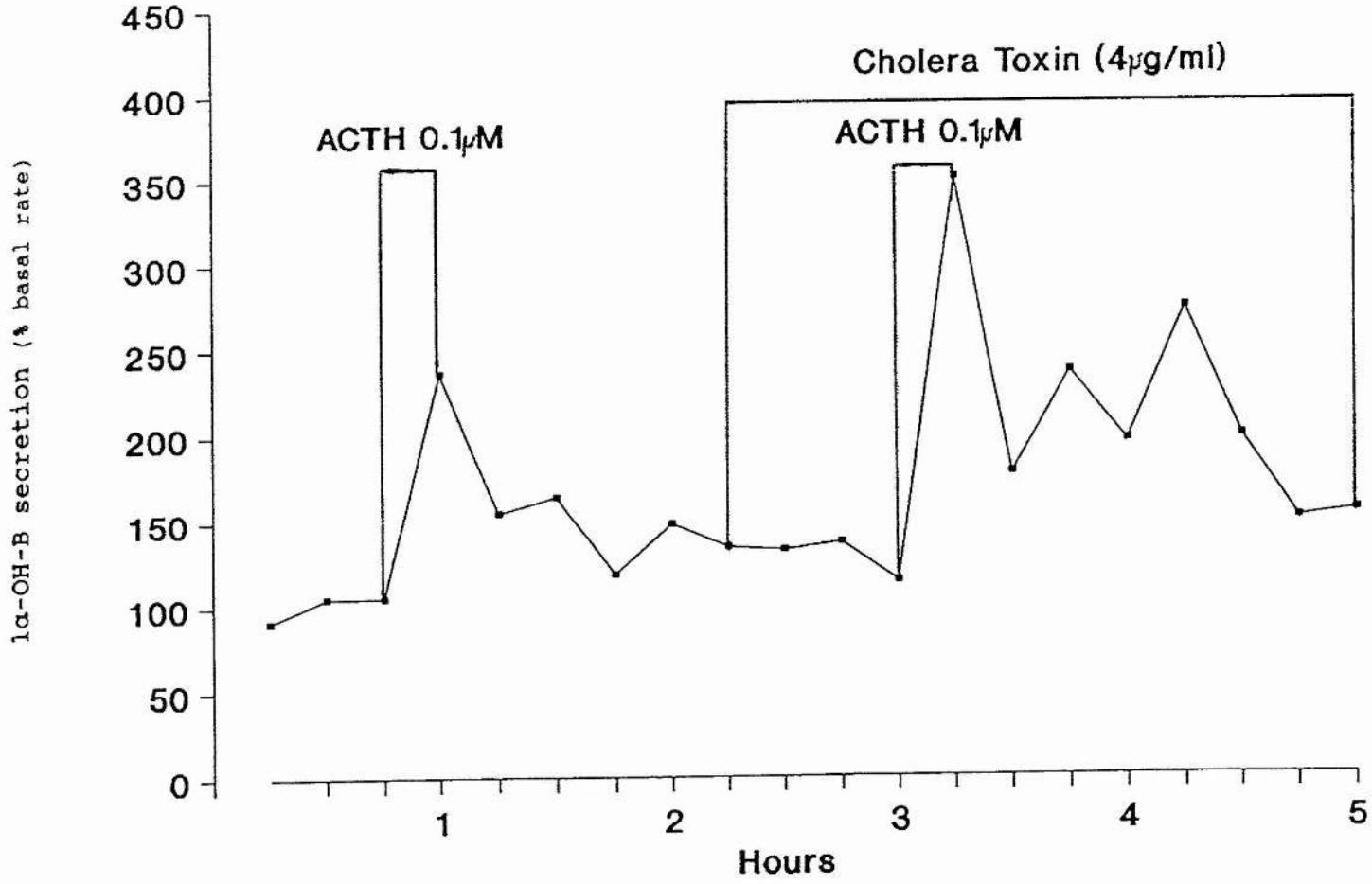
4.7.6 Effect of porcine ACTH in the presence of cholera toxin

In the presence of cholera toxin (4μg/ml) the response to 0.1μM porcine ACTH was significantly increased ($p<0.05$; n=3) to 253% ± 17% (S.E.M.) above basal 1α-OH-B secretory rate (Figure 4.17). The duration of response was increased to over 2 hours and secretion of 1α-OH-B did not return to a steady-state basal level (not shown). These results suggest that the steroidogeneic effect of ACTH is mediated through G proteins.

The results of the current study suggest that ACTH-induced steroidogenesis in dogfish, as in mammals and other vertebrates, occurs via G protein-mediated activation of adenylate cyclase. Generation of the intracellular messenger, cAMP, by adenylate cyclase activates a cascade

Figure 4.17

Fig. 4.17 Effect of ACTH in the Presence of Cholera Toxin
Time course showing the effect of 0.1μM ACTH on 1α-hydroxycorticosterone (1α-OH-B) production in the absence and presence of cholera toxin (4μg/ml). 1α-OH-B production is expressed as a percentage of basal secretory rate (n=3). Mean basal secretory rates were 503 ± 71, 1031 ± 109, and 773 ± 96 fmol/mg per 15 mins.


Cholera Toxin (4μg/ml)
ACTH 0.1μM
ACTH 0.1μM
1α-OH-B secretion (% basal rate)
450
400
350
300
250
200
150
100
50
0
1
2
3
4
5
Hours

system of enzymes which leads to increased 1α-OH-B production.

4.7.7 Effect of Ca^{2+}-free perifusion medium

Immediately following changeover to Ca^{2+}-free medium there was a significant increase ($p<0.01$, $n=8$,) in 1α-OH-B production to 261 ± 27% (S.E.M.) above basal and this was typically of 30-45 minutes duration, whereafter normal basal secretory rate was resumed (Figure 4.18 and 4.22). This phenomenon has also been observed in amphibian interrenal gland preparations (Lihrmann et al, 1985, 1987).

4.7.8 Effect of porcine ACTH in normal Ca^{2+}-free medium

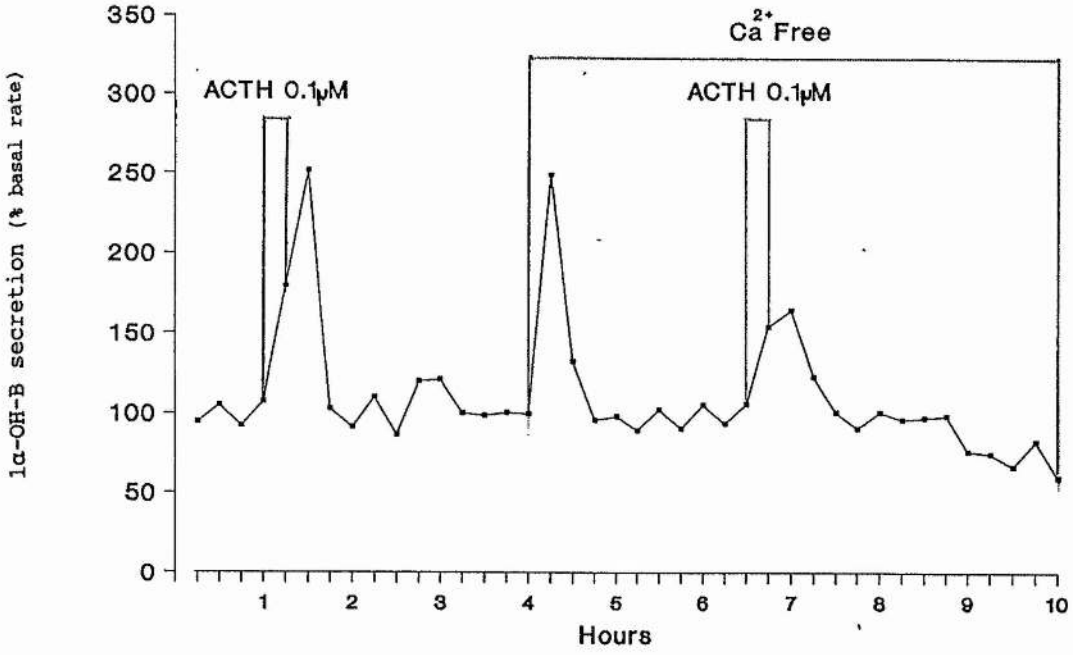
The response to 0.1μM porcine ACTH was significantly reduced ($p<0.01$) from 198 ± 17% (S.E.M.) to 47 ± 6% (S.E.M.) above 1α-OH-B basal secretory rate when Ca^{2+} was absent from the perifusion medium (Figure 4.18). The duration of response to 0.1μM porcine ACTH was unchanged. In mammals and other vertebrates the presence of external Ca^{2+} is required for the binding of ACTH to its receptor and this also appears to be true in dogfish.

4.7.9 Effect of 50μM dantrolene

Dantrolene (1-[{5-[p-nitrophenyl] furfurylidene} amino] hydantoin) is believed to inhibit the release of calcium from intracellular stores (Lihrmann et al, 1987). Dantrolene was initially dissolved in 0.2% DMSO, which does not affect basal steroid secretion. The presence of 50μM dantrolene resulted in a significant reduction ($p<0.001$, $n=8$) in 1α-OH-B basal secretion rate of 20 ± 3% (S.E.M.) (Figures 4.19 and 4.23). Normal basal secretion rate had

Figure 4.18

Fig. 4.18 Response to ACTH in Calcium-Free Media
Effect of 0.1μM porcine ACTH on 1α-hydroxycorticosterone (1α-OH-B) production by dogfish interrenal glands in the presence and absence of Ca^{2+} in the perifusion media.
(a) Representative time course showing the responses to 0.1μM porcine ACTH in the presence of normal and of Ca^{2+}-free perifusion medium. Note the characteristic spike of 1α-OH-B production immediately following change to Ca^{2+}-free media.
(b) Comparative effect of 0.1μM porcine ACTH on 1α-OH-B production in the presence and absence of external Ca^{2+}. Results are means ± S.E.M. (n=4) of 1α-OH-ß production expressed as percentage increases above basal secretory rates.
Mean basal secretory rates were 1486 ± 74, 7616 ± 896, 2620 ± 383 and 2561 ± 146 fmol/mg per 15 min.
** $p<0.01$ represents a significantly decreased response compared to the effect in normal media (Student's t-test).


1α-OH-B secretion (% basal rate)
350
300
250
200
150
100
50
0
ACTH 0.1μM
Ca2+ Free
ACTH 0.1μM
1
2
3
4
5
6
7
8
9
10
Hours

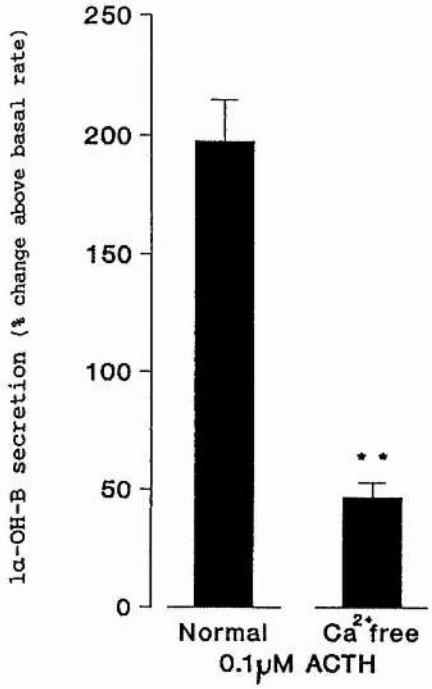
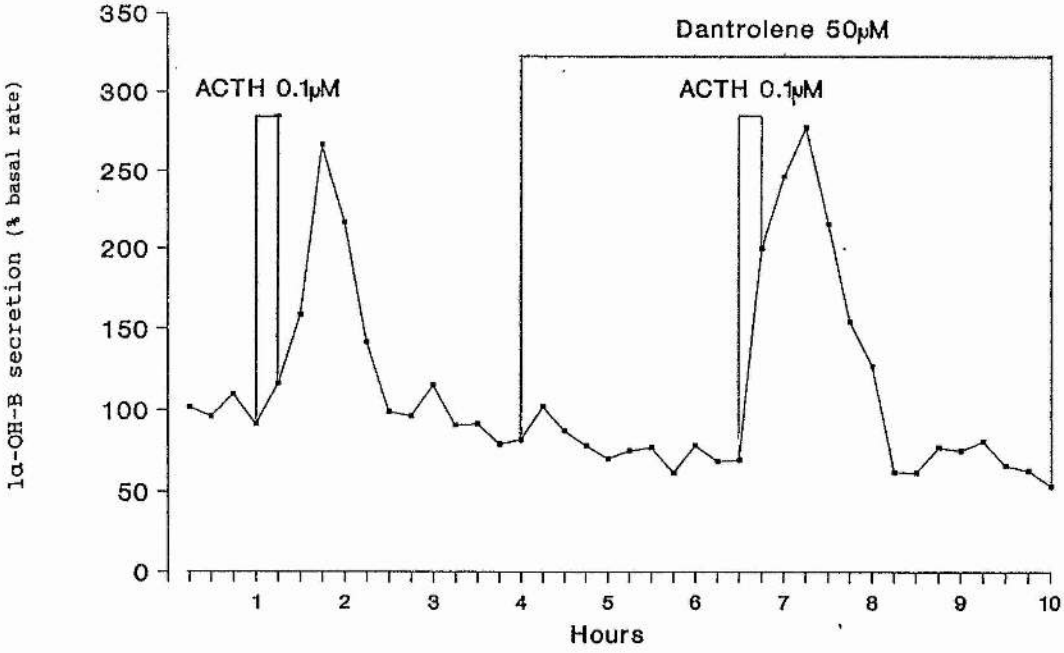
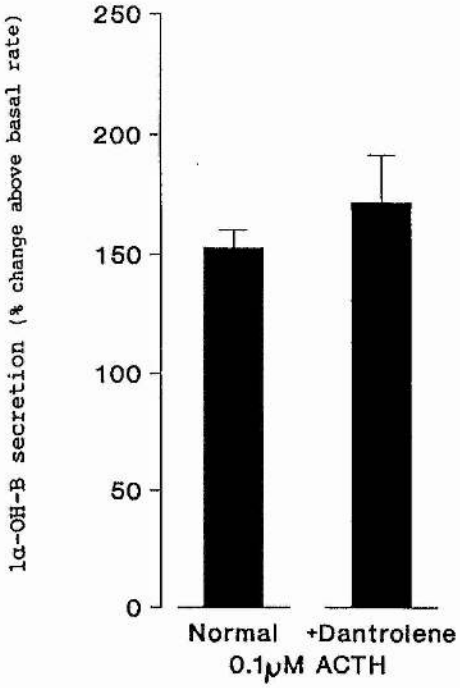

1α-OH-B secretion (% change above basal rate)
250
200
150
100
50
0
* *
Normal
Ca2+ free
0.1μM ACTH

Figure 4.19

Fig. 4.19 Response to ACTH in the Presence of Dantrolene
Effect of 0.1μM porcine ACTH on 1α-hydroxycorticosterone (1α-OH-B) production by dogfish interrenal glands in the absence and presence of 50μM dantrolene.
(a) Representative time course showing the responses to 0.1μM ACTH before and after the addition of dantrolene. 1α-OH-B production is expressed as a percentage of basal secretory rate.
(b) Comparative effect of 0.1μM ACTH on 1α-OH-B production in the absence and presence of 50μM dantrolene. Results are means ± S.E.M. (n=4) of 1α-OH-ß production expressed as percentage increases above basal secretory rates.
Mean basal secretory rates were 1043 ± 88, 406 ± 16, 379 ± 36 and 555 ± 52 fmol/mg per 15 min.
No statistically significant differences were observed.


Dantrolene 50μM
ACTH 0.1μM
ACTH 0.1μM
1α-OH-B secretion (% basal rate)
350
300
250
200
150
100
50
0
1
2
3
4
5
6
7
8
9
10
Hours


1α-OH-B secretion (% change above basal rate)
250
200
150
100
50
0
Normal
+Dantrolene
0.1μM ACTH

still not been restored two hours after changing back to normal perifusion medium (not shown). The depressor effect of dantrolene on basal corticosteroid secretion has also been observed in amphibia (Lihrmann et al, 1987) and may be a non-specific effect on calcium homeostasis. Dantrolene has also been shown to inhibit the stimulated release of calcium from intracellular stores (Francis, 1979; Kojima, Kojima and Rasmussen, 1985a; Lihrmann et al, 1987).

4.7.10 Effect of porcine ACTH in the presence of 50μM dantrolene

The presence of 50μM dantrolene had no significant effect on the response to 0.1μM porcine ACTH (Figure 4.19). This result suggests that the steroidogenic effect of ACTH in dogfish may not involve the mobilisation of Ca^{2+} from intracellular Ca^{2+} stores.

4.7.11 Effect of 10μM verapamil

Verapamil (10μM) had no apparent effect on 1α-OH-B basal secretion rate nor on the response to 0.1μM porcine ACTH which was unchanged. Verapamil blocks voltage-dependent calcium channels and these results suggest that corticosteroid basal secretory rate in elasmobranchs is not significantly mediated by these channels at least in vitro.

The response to porcine ACTH was apparently unaffected by verapamil, suggesting that ACTH-induced corticosteroidogenesis may not be significantly mediated through voltage dependent calcium channels. This is in contrast to amphibia where verapamil induced a dose-related inhibition of ACTH-induced corticosteroidogenesis (Lihrmann et al, 1986).

4.7.12 Effect of increasing doses of Val^5-AII

Figure 4.20 shows the effect of increasing doses (0.01-1μM) of Val^5-AII on 1α-OH-B production. The responses were dose-dependent with a maximal increase in 1α-OH-B production of 176 ± 19% (S.E.M.) above basal secretory rate ($p<0.01$). The response to Val^5-AII was rapid, the peak response occurring within 15-30 minutes of stimulation. The duration of response increased with dose and ranged from 1-1½ hours following a maximal dose of 1μM Val^5-AII. The presence of an endogenous elasmobranch AII-like-peptide has been suggested (Hazon, Balment, Perrott and O'Toole, 1989) and it is reasonable to assume that it would be a more potent stimulant of steroidogenesis than the one used in this study.

4.7.13 Comparison of responses to Val^5- and Ile^5-AII

Both Val^5-AII and Ile^5-AII (0.1μM) significantly increased ($p<0.05$ and $p<0.01$, respectively) the secretion rate of 1α-OH-B above basal (Figure 4.21). Ile^5-AII was significantly more potent than Val^5-AII ($p<0.005$) producing a stimulation above basal of 393 ± 44% (S.E.M.) compared with 121 ± 13% (S.E.M.) produced by Val^5-AII. Ile^5-AII was used in preference to Val^5-AII in subsequent experiments.

The response to Ile^5-AII appeared typically to be biphasic, the peak response occurring within 15-30 minutes of stimulation followed by a second, smaller peak or plateau within 30-60 minutes later. The duration of response following a dose of 0.1μM Ile^5-AII was typically 2-2½ hours.

Figure 4.20

Fig. 4.20 Effect of Increasing Doses of Val^5-Angiotensin II (Val^5-AII) on 1α-hydroxycorticosterone production

Each interrenal gland was allowed 2h to recover between each challenge. Results are means ± S.E.M. (n=5) of 1α-OH-B production expressed as percentage increases above basal secretory rates. Mean basal secretory rates were:
(i) for 0.01μM Val^5-AII, 689 ± 52, 1162 ± 71, 1412 ± 68, 763 ± 90 and 1350 ± 125 fmol/mg per 15 mins;
(ii) for 0.1μM Val^5-AII, 2722 ± 379, 2752 ± 188, 5020 ± 115, 4432 ± 342 and 465 ± 18 fmol/mg per 15 min;
(iii) for 1.0μM Val^5-AII, 156 ± 37, 139 ± 36, 836 ± 92, 927 ± 45 and 1000 ± 126 fmol/mg per 15 mins.
* $p<0.05$, ** $p<0.01$ compared with basal secretory rate (Student's t-test).

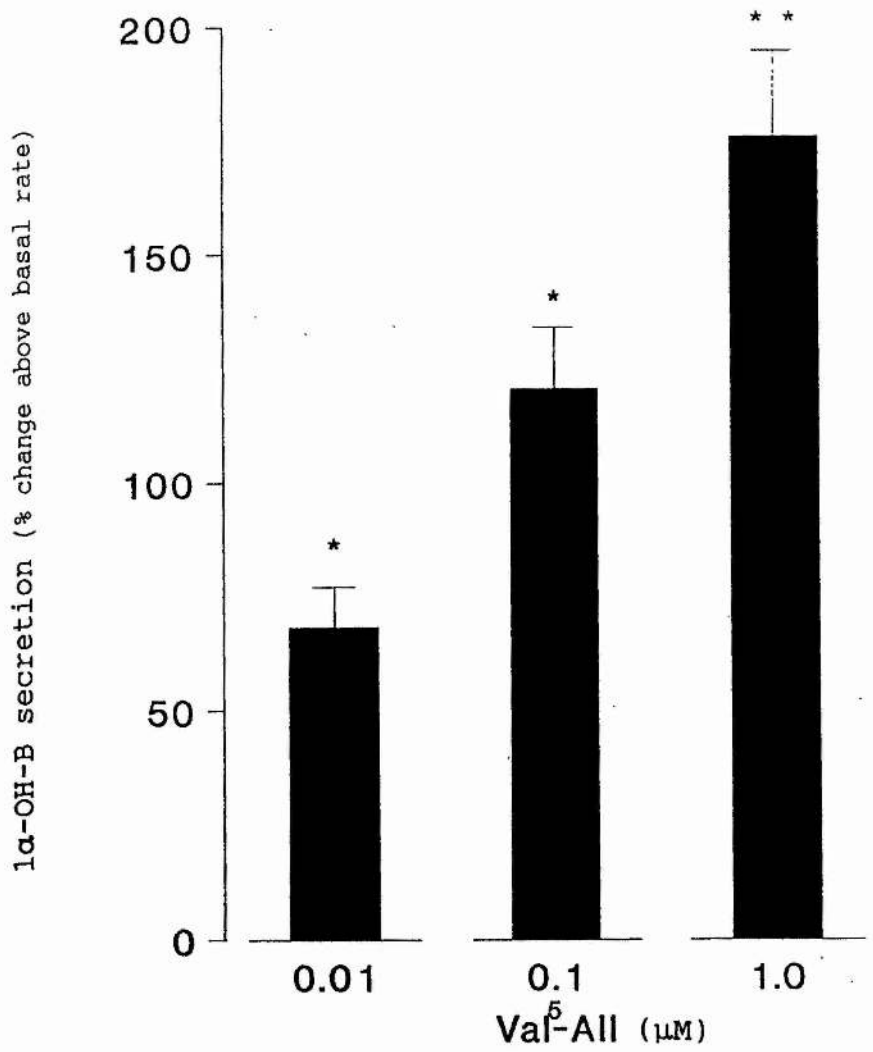
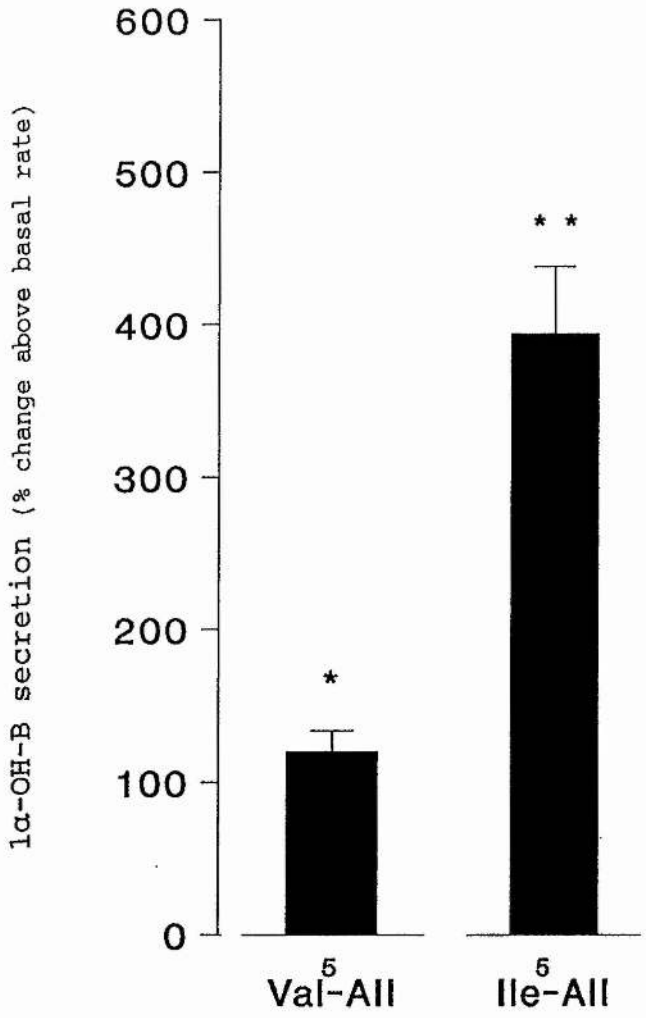

1α-OH-B secretion (% change above basal rate)
200
150
100
50
0
* *
*
*
0.01
0.1
1.0
Val5-AII (μM)

Figure 4.21

Fig. 4.21 Comparison of Val^5- and Ile^5-Angiotensin II
Comparative effects of Val^5-Angiotensin II (Val^5-AII) and Ile^5-AII (both 0.1μM) on 1α-hydroxycorticosterone production by perifused dogfish interrenal glands. Results are means ± S.E.M. (n=5) of 1α-OH-B production expressed as percentage increases above basal secretory rate. Mean basal secretory rates for 0.1μM Val^5-AII are given in the heading for Figure 5.
Mean basal secretory rates for 0.1μM Ile^5-AII were 311 ± 61, 598 ± 142, 1363 ± 179, 616 ± 114 and 1296 ± 212 fmol/mg per 15 min.
* $p<0.05$, ** $p<0.01$ compared with basal secretory rate (Student's t-test).


1α-OH-B secretion (% change above basal rate)
600
500
400
300
200
100
0
*
* *
5
Val-AII
5
Ile-AII

4.7.14 Effect of Ile^5-AII in normal and Ca^{2+}-free medium

The response to 0.1μM Ile^5-AII was significantly reduced ($p<0.01$) from 438 ± 49% (S.E.M.) to 115 ± 18% (S.E.M.) above 1α-OH-B basal secretory rate in Ca^{2+}-free perifusion medium (Figure 4.22). The duration of response to 0.1μM Ile^5-AII appeared to be unchanged. Attenuation of the steroidogenic response to AII stimulation in calcium-free media has also been demonstrated in mammals and amphibians (Kojima, Kojima and Rasmussen, 1985a,c; Lihrmann et al, 1986, 1987).

4.7.15 Effect of Ile^5-AII in the presence of 50μM dantrolene

The response to 0.1μM Ile^5-AII was significantly reduced ($p<0.01$) in the presence of 50μM dantrolene from 389 ± 47% (S.E.M.) to 196 ± 29% (S.E.M.) above basal secretory rate (Figure 4.23). The duration of response to 0.1μM Ile^5-AII was unchanged as was the time to peak response.

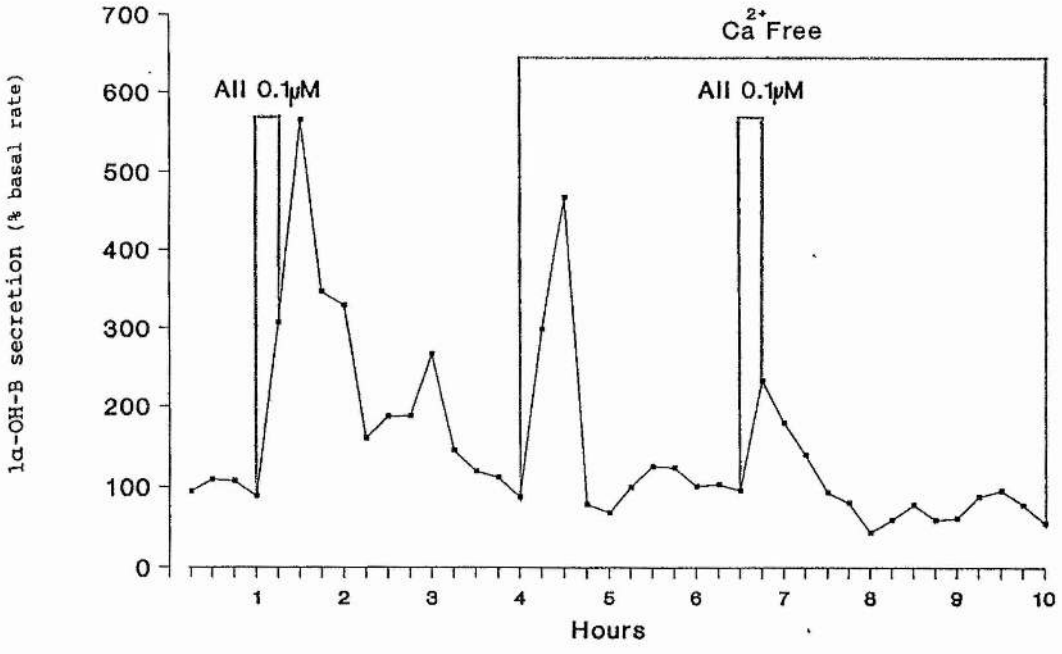
This result indicates clearly that AII-induced-steroidogenesis in dogfish involves the mobilisation of intracellular Ca^{2+} from intracellular stores as it does in amphibians (Lihrmann et al, 1987).

4.7.16 Effect of Ile^5-AII in the presence of 10μM verapamil

Verapamil (10μM) had no effect on the response to 0.1μM Ile^5-AII suggesting that AII-induced steroidogenesis is not significantly mediated via voltage-dependent calcium

Figure 4.22

Fig. 4.22 Response to Ile^5-AII in Calcium-Free Media
Effect of 0.1μM Ile^5-Angiotensin II (Ile^5-AII) on 1α-hydroxycorticosterone (1α-OH-B) production by dogfish interrenal glands in the presence and absence of Ca^{2+} in the perifusion media.
(a) Representative time course showing the responses to 0.1μM Ile^5-AII in the presence of normal and Ca^{2+}-free perifusion media. A characteristic spike of 1α-OH-B production is evident immediately following change to Ca^{2+}-free media.
(b) Comparative effect of 0.1μM Ile^5-AII on 1α-OH-B production in the presence and absence of external Ca^{2+}. Results are means ± S.E.M. (n=4) of 1α-OH-ß production expressed as percentage increases above basal secretory rates.
Mean basal secretory rates were 1611 ± 11, 8654 ± 962, 2163 ± 120 and 2320 ± 174 fmol/mg per 15 min.
*** $p<0.001$ represents a significantly decreased response compared to the effect in normal media (Student's t-test).


700
600
500
400
300
200
100
0
1α-OH-B secretion (% basal rate)
AII 0.1μM
Ca2+ Free
AII 0.1μM
1
2
3
4
5
6
7
8
9
10
Hours

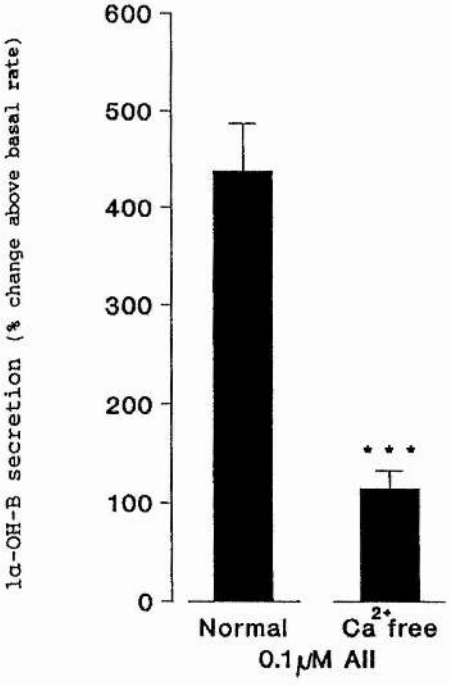

600
500
400
300
200
100
0
1α-OH-B secretion (% change above basal rate)
* * *
Normal
Ca2+ free
0.1μM AII

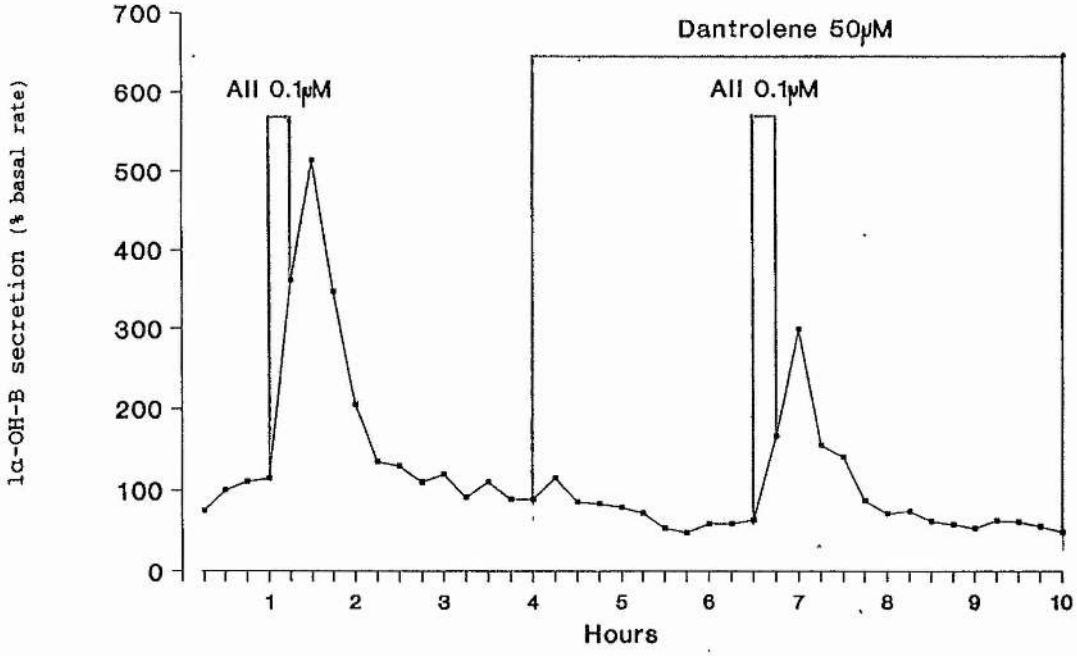
Figure 4.23

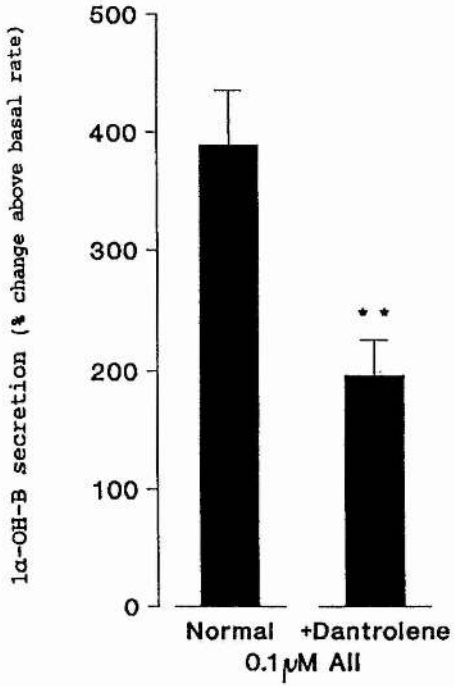
Fig. 4.23 Response to Ile^5-AII in the Presence of Dantrolene

Effect of 0.1μM Ile^5-Angiotensin II (Ile^5-AII) on 1α-hydroxycorticosterone (1α-OH-B) production by dogfish interrenal glands in the absence and presence of 50μM dantrolene.

(a) Representative time course showing the responses to 0.1μM Ile^5-AII before and after the addition of dantrolene. 1α-OH-B secretion is expressed as a percentage of basal secretory rate.

(b) Comparative effect of 0.1μM Ile^5-AII on 1α-OH-B production in the absence and presence of 50μM dantrolene. Results are means ± S.E.M. (n=4) of 1α-OH-B production expressed as percentage increases above basal secretory rates. Mean basal secretory rates were 1569 ± 36, 353 ± 21, 573 ± 32 and 2066 ± 182 fmol/mg per 15 min. *** $p<0.01$ represents a significantly decreased response compared to the effect in normal media (Student's t-test).


1α-OH-B secretion (% basal rate)
700
600
500
400
300
200
100
0
AII 0.1μM
Dantrolene 50μM
AII 0.1μM
1
2
3
4
5
6
7
8
9
10
Hours


1α-OH-B secretion (% change above basal rate)
500
400
300
200
100
0
**
Normal
+Dantrolene
0.1μM AII

channels in dogfish. In amphibia AII-induced steroidogenesis was slightly inhibited but not abolished in the presence of verapamil (Lihrmann et al, 1986).

4.7.17 Effect of Ca^{2+} ionophore A23187

50μM A23187 produced a significant increase ($p<0.05$, $n=4$) in 1α-OH-B secretion to 80 ± 13% (S.E.M.) above basal. Response was evident immediately and peaked within 30-45 minutes after administration. The overall duration of response was approximately 75-90 minutes.

The Ca^{2+} ionophore, A23187 has been widely used because of its ability to traverse a lipid bilayer and permit the introduction of Ca^{2+} into cells and organelles.

The above result demonstrates that a Ca^{2+} influx is capable of stimulating steroidogenesis in dogfish interrenal cells. Taken as a whole, the perifusion studies on AII-induced steroidogenesis have shown that stimulation involves Ca^{2+} influx through non-voltage dependent Ca^{2+} channels and the mobilisation of Ca^{2+} from intracellular stores. In mammals and other vertebrates this process is controlled by the IP_3/DG second messenger system and it is probable that AII-induced steroidogenesis in dogfish is under similar control.

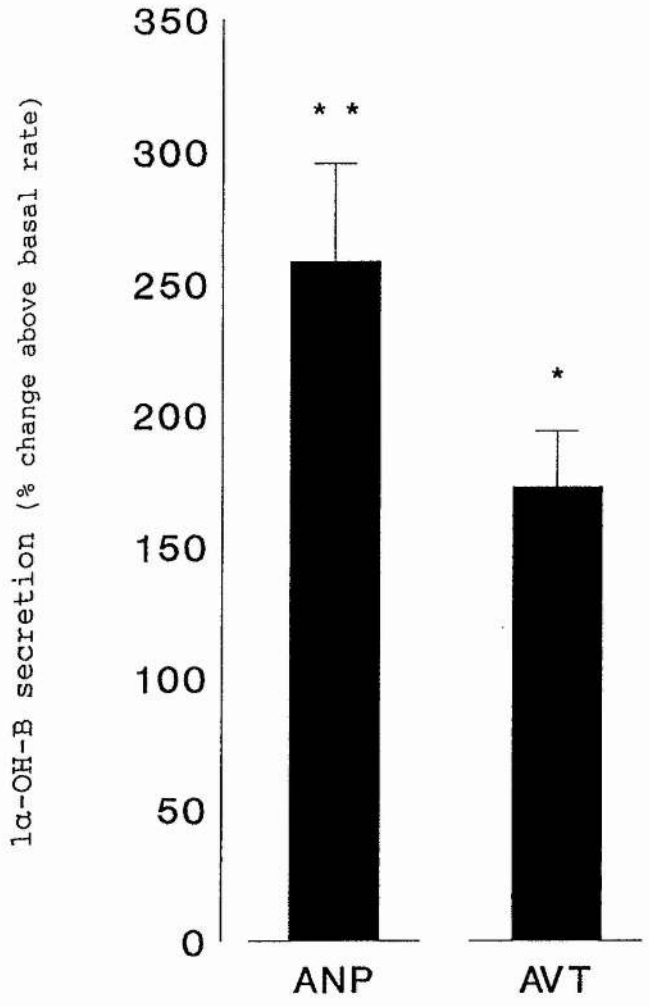
4.7.18 Comparison of responses to ANP and AVT

Doses of ANP and AVT (both 1.0μM) significantly increased ($p<0.01$ and $p<0.05$, respectively) the secretion rate of 1α-OH-B above basal (Figure 4.24). ANP was more potent than AVT ($p<0.05$) producing a stimulation above

Figure 4.24

Fig. 4.24 Comparison of Atrial Natriuretic Peptide and Arginine Vasotocin

Comparative effects of atrial natriuretic peptide (ANP) and arginine vasotocin (AVT) (both 1.0μM) on 1α-hydroxycorticosterone (1α-OH-B) production by perifused dogfish interrenal glands.
Results are means ± S.E.M. (n=4) of 1α-OH-B production expressed as percentage increases above basal secretory rates.
Mean basal secretory rates for 1.0μM ANP were 888 ± 142, 440 ± 29, 673 ± 50 and 290 ± 12 fmol/mg per 15 mins.
Mean basal secretory rates for 1.0μM AVT were 742 ± 40, 943 ± 80, 213 ± 19 and 680 ± 30 fmol/mg per 15 mins.
* $p<0.05$, ** P,0.01 compared with basal secretory rate (Student's t-test).


1α-OH-B secretion (% change above basal rate)
350
300
250
200
150
100
50
0
* *
*
ANP
AVT

basal of 258 ± 37% (S.E.M.) compared with 172 ± 21% (S.E.M.) produced by AVT.

4.7.19 Effect of dibutyryl cGMP

Dibutyryl cGMP (10pM-10μM) produced a dose dependent increase in 1α-OH-B production (Figure 4.25), with a maximum response of 285 ± 24% (S.E.M.) above basal secretory rate ($p<0.01$).

Responses to dibutyryl cGMP were typically biphasic, the peak response occurring within 30 minutes of stimulation followed by a secondary peak approximately 1-1½ hours later which was smaller in magnitude. Between peaks 1α-OH-B production remained significantly elevated above basal. The duration of response varied with dose and ranged from 2-3 hours following a maximal dose of 10μM dibutyryl cGMP. It is well known that the effects of ANP are mediated via the guanylate cyclase/cGMP second messenger system and the responses to ANP and dibutyryl cGMP obtained in this study suggest that this may also be the case in dogfish.

4.7.20 Effect of increasing concentrations of potassium ions

Figure 4.26 shows the effect of increasing the potassium ion concentration of the perifusion medium from 8mM to 12,18 and 28mM. A significant response was only evident at 28mM which resulted in an increase in 1α-OH-B production of 155 ± 33% (S.E.M.) above basal secretory rate ($p<0.01$). A further increase in potassium ion concentration to 40mM had no significant effect on basal

Figure 4.25

Fig. 4.25 Effect of Increasing Doses of Dibutyryl Cyclic GMP on 1α-hydroxycorticosterone Production
Each interrenal gland was allowed 2h recovery between each challenge. Results are means ± S.E.M. (n=4) of 1α-OH-B production expressed as percentage increases above basal secretory rates. Mean basal secretory rates were:
(i) for 0.00001μM dibutyryl cGMP 394 ± 10, 1314 ± 202, 1173 ± 79 and 876 ± 111 fmol/mg per 15 min;
(ii) for 0.001μM dibutyryl cGMP 743 ± 372, 1086 ± 32, 1200 ± 99 and 172 ± 38 fmol/mg per 15 min;
(iii) for 0.1μM dibutyryl cGMP 926 ± 56, 1433 ± 164, 796 ± 74 and 475 ± 45 fmol/mg per 15 min;
(iv) for 10μM dibutyryl cGMP 1332 ± 20, 1468 ± 323, 809 ± 25 and 1653 ± 141 fmol/mg per 15 min.
* $p<0.05$, ** $p<0.01$ compared with basal secretory rate (Student's t-test).

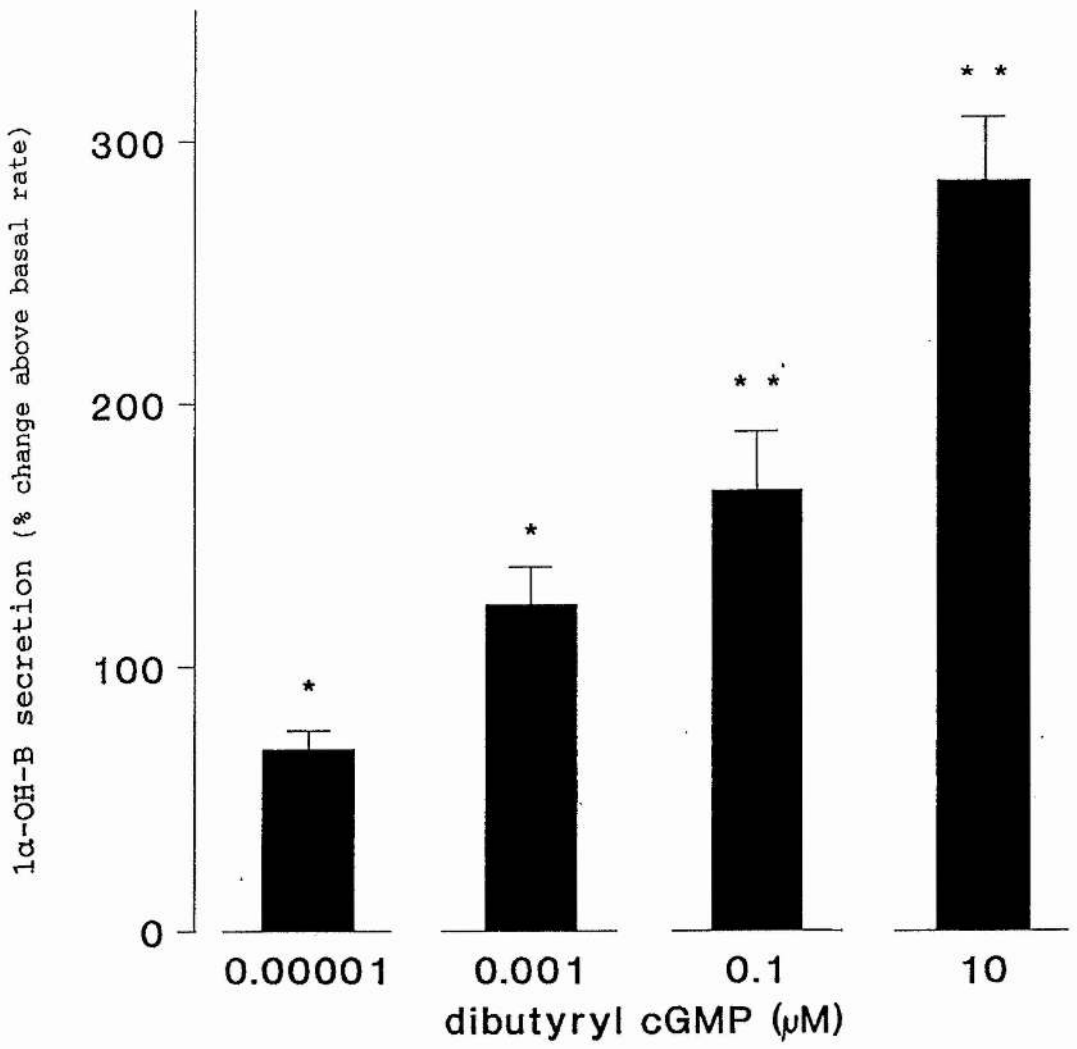
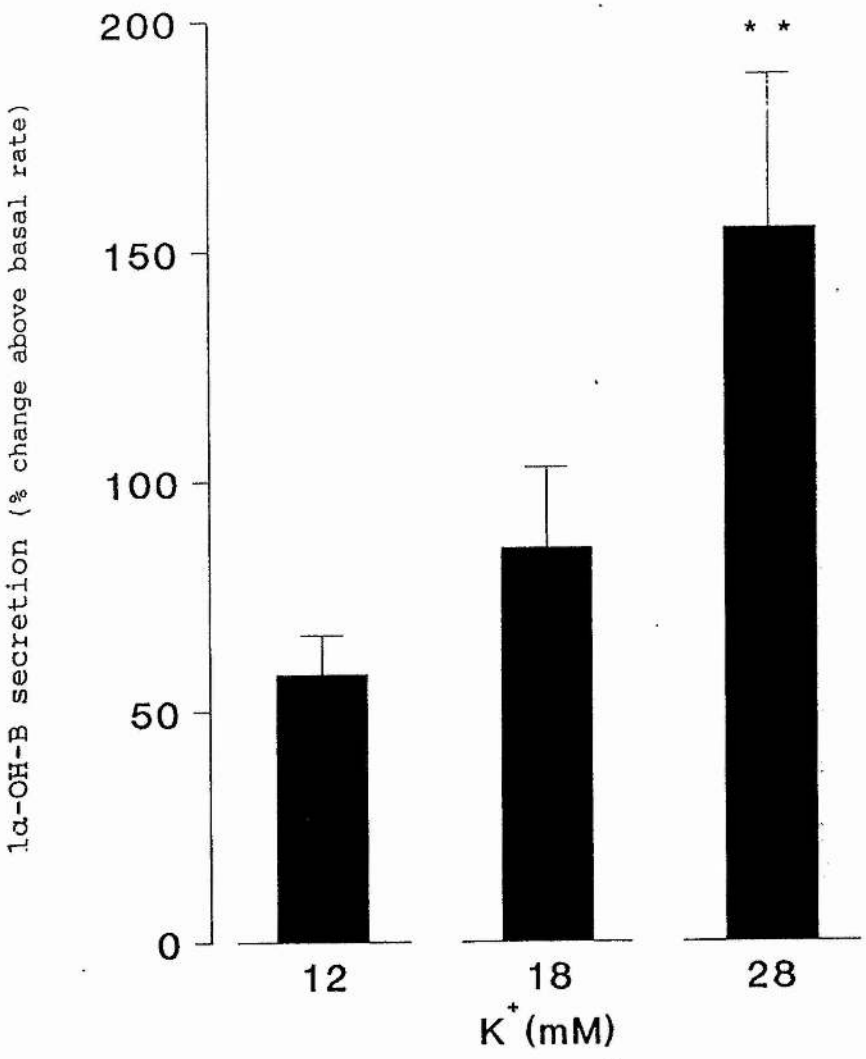

1α-OH-B secretion (% change above basal rate)
300
200
100
0
* *
* *
*
*
0.00001
0.001
0.1
10
dibutyryl cGMP (μM)

Figure 4.26

Fig. 4.26 Effects of Increasing Doses of K^+ Ions on 1α-hydroxycorticosterone Production
Each interrenal gland was allowed 2h recovery between each challenge. Results are means ± S.E.M. (n=4) of 1α-OH-B production expressed as percentage increases above basal secretory rates. Mean basal secretory rates were:
(i) for 12mM K^+, 3564 ± 238, 1926 ± 205, 3699 ± 628 and 4783 ± 899 fmol/mg per 15 min;
(ii) for 18mM K^+, 3902 ± 206, 2420 ± 413, 2848 ± 374 and 7003 ± 344 fmol/mg per 15 min;
(iii) for 28mM K^+, 3496 ± 178, 4375 ± 272, 4753 ± 211 and 7732 ± 762 fmol/mg per 15 min;
* $p<0.01$ compared with basal secretory rate (Student's t-test).


1α-OH-B secretion (% change above basal rate)
200
150
100
50
0
* *
12
18
28
K+ (mM)

secretory rate (not shown). The response to 28mM K^+ was immediate and short lived. K^+ is a well known activator of adrenocortical steroidogenesis in other vertebrate groups.

4.7.21 Effect of increasing concentrations of sodium ions

The presence of increasing concentrations of sodium ions (320, 360 and 400mM) in the perifusion medium produced slight perturbations, but had no significant effect on the basal secretory rate of 1α-OH-B (n=4).

This experiment was carried out primarily to investigate whether the very high plasma sodium levels observed in LPD fish in 130% seawater were capable of stimulating steroidogenesis directly in the dogfish interrenal gland and clearly, this is not the case. Experiments carried out by O'Toole (1987) have shown that increasing the osmolality of the perifusion medium (by addition of the inert osmolyte, mannitol) or decreasing the omolality of the perifusion medium (by dilution of distilled water) also had no effect on basal secretion rate of 1α-OH-B.

It is clear from these perifusion experiments that the elevated plasma 1α-OH-B concentrations measured in osmotically stressed fish were not the result of direct interrenal stimulation by changes in plasma osmolality or electrolyte concentrations, and were probably hormonally mediated.

5. DISCUSSION

It is well known that elasmobranchs can alter their blood osmolality in response to changing environmental osmolality. In dilute seawater plasma osmolality decreases and in concentrated seawater it increases (Scott, 1913; Duval, 1925: Chaisson, 1930: Margaria, 1931). Elasmobranchs cannot tolerate abrupt transfer from one medium to another and in most early studies death resulted within one to six hours.

Studies employing stepwise and gradual adaptation have revealed that a number of purely marine species will survive in waters between 300 and 500 mOsmol/kg. To date, the responses of all species investigated include a reduction in total plasma osmolality with concomitant reductions in plasma sodium, chloride and urea concentrations (Price, 1967: Price and Creaser, 1967: Goldstein, Oppelt and Maren, 1968: Goldstein and Forster, 1971: de Vlaming and Sage, 1973: Chan and Wong, 1977: Mandrup-Poulsen, 1981: Hazon and Henderson, 1984). Normally the relative loss of urea is greater than that of sodium and chloride, although in the stingray, Dasyatis sabina, the hypo-osmolality of the plasma seems to result mainly from hypochloraemia and hyponatraemia (de Vlaming and Sage, 1973). The decrease in plasma urea concentration when fish enter lower environmental salinities may result from increased renal excretion and/or reduced hepatic synthesis and there is variation between species (Goldstein, Oppelt and Maren, 1968: Goldstein and Forester, 1971: Wong and Chan, 1977: Hazon and Henderson, 1984). The

regulation of water balance and blood volume during exposure to progressively more dilute environments also varies with species, Dasyatis sabina (de Vlaming and Sage, 1973), Negaprion brevirostris (Goldstein, Oppelt and Maren, 1968) and Scyliorhinus canicula (Hazon and Henderson, 1984) all maintained normal haematocrits and body weights if given sufficient time to adapt to dilute environments. However, in Squalus acanthias, haematocrit decreased by one third upon exposure to 70% seawater (Forster, Goldstein and Rosen, 1972). Hemiscyllium gained up to 40% of its body weight in 12% seawater(Chan and Wong, 1977). The latter study is difficult to relate to others reported in the literature because the weight changes reported were achieved at an extremely low environmental osmolality. Adaptation of elasmobranchs to environments more concentrated than normal seawater produced broadly opposite effects on plasma composition to those seen upon exposure to more dilute conditions (Price and Creaser, 1967: Haywood, 1973: Mandrup-Poulsen, 1981: Hazon and Henderson, 1984). In these studies clear differential effects were evident in plasma urea, sodium and chloride concentrations.

In the early adaptation studies, prior to those of Hazon and Henderson (1984) the rapidity with which investigators adapted their experimental animals varied considerably. Together with the obvious species variations it is difficult to directly compare the results of those studies with those of the present study. In the present studies on Scyliorhinus canicula the environmental

adaptation protocol followed was similar to that of Hazon and Henderson (1984) and allowed sufficient time for the gradual and complete acclimation of the animals to either increased or decreased seawater osmolalities.

Fish on both the HPD and LPD showed no significant change in body weight during the initial 30 day adaptation period, Starved fish over the same period showed a large and significant decrease in body weight (O'Toole and Hazon, unpublished observation). No significant differences in plasma concentrations of total lipid or cholesterol were evident between the two dietary groups. The higher lipid content of the LPD did not, therefore, appear to increase plasma lipid concentration to a level above that seen in the HPD fish, perhaps indicating that the excess dietary lipid was not completely absorbed at the gut. Plasma cholesterol concentration varied directly with changes in plasma osmolality and was not related to changes in plasma corticosteroid levels. This important observation clearly showed that the LPD was not influencing plasma steroid concentration by providing a potentially greater source of substrate for steroid synthesis.

Elasmobranchs have two main sources of substrate for urea synthesis: ammonia release from muscle and dietary protein intake (Leech, Goldstein, Cha and Goldstein, 1979). The former can account for a significant proportion of urea synthesis and depends to a certain extent on the intake of dietary protein. Decreases in muscle protein due to the release of ammonia are replaced by dietary protein intake.

During starvation elasmobranch body weight decreases as a result of the continued loss of muscle protein as ammonia (Leech, Goldstein, Cha and Goldstein, 1979). Ammonia release from muscle, however, also shows an initial decrease after which the rate remains constant at a lower, but significant level. As a consequence of the decrease in ammonia release, hepatic urea production is also decreased (O'Toole and Hazon, unpublished observation) (Figure 5.1) and this eventually leads to a decrease in plasma urea concentration in starved fish (Leech, Goldstein, Cha and Goldstein, 1979).

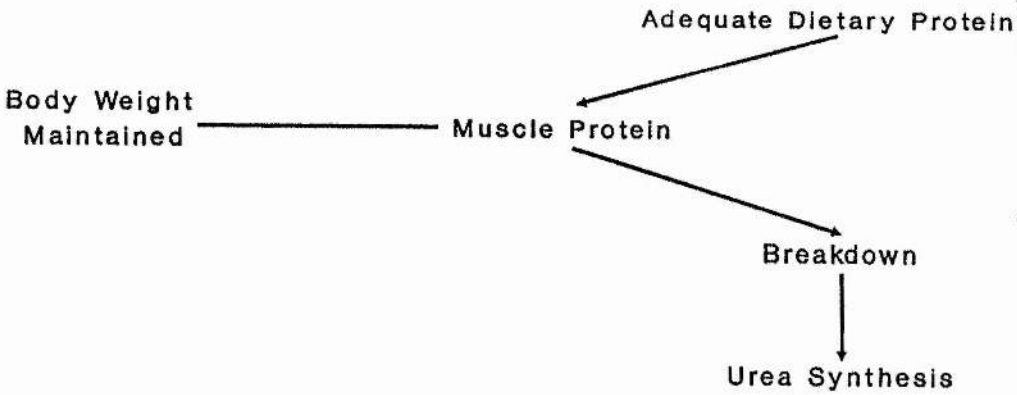
Plasma protein concentrations were not significantly different between the two protein diet groups. With hindsight it is clear that these experiments reveal little of the destination of dietary protein following its digestion and absorption at the gut or of the rate of muscle protein breakdown. Perhaps a better experiment would have been to formulate high and low protein diets containing a high specific activity of a particular radioactive amino acid and measure the absorption and distribution kinetics of that amino acid throughout the different body compartments of the dogfish. This would be a complex procedure but would enable differences to be detected between the two dietary groups in specific amino acid incorporation into muscle and other tissues .

The HPD fish used in this study maintained body weight and hepatic urea production and were apparently adequately fed (Figure 5.1). However, fish on a LPD, while

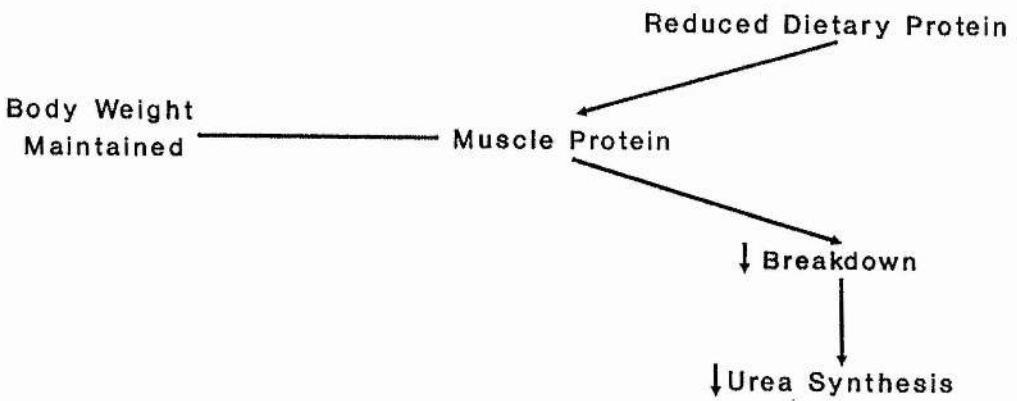
Figure 5.1

Fig. 5.1 Interrelationship of dietary protein intake and urea metabolism.

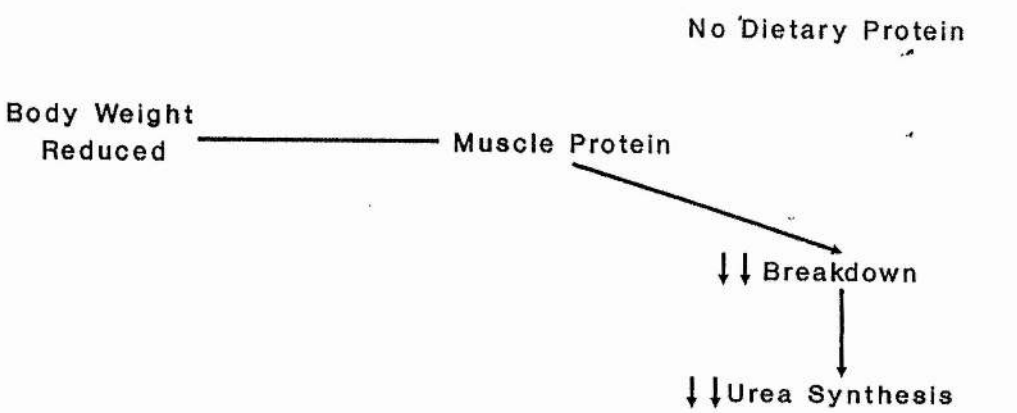
HIGH PROTEIN DIET



LOW PROTEIN DIET



STARVED



maintaining body weight exhibited a significant decrease in hepatic urea production. LPD fish thus appear to be intermediate between adequately fed and starved fish; the dietary protein content of the LPD is apparently sufficient to replace the breakdown of muscle protein (i.e. maintain body weight) but is insufficient to maintain the level of urea synthesis observed in HPD fish. This is a direct result of dietary restriction of substrates for urea synthesis but may also reflect a dietary-induced decrease in the rate of ammonia release from muscle tissue (Figure 5.1). Starved fish were not used in this study because they were unable to tolerate adaptation to environments of increased osmolality (O'Toole and Hazon, unpublished observation).

The following discussion will present an hypothesis on the mechanisms by which the elasmobranchs osmoregulate under conditions of high and low dietary protein intake, based primarily on the results obtained in this study and on the reported actions of vertebrate hormones. Where direct experimental evidence for changes in endocrine activity or osmoregulatory organ function is not available this has been indicated in the text.

Effects on osmoregulation in 100% seawater

The protein content of the HPD was found to be adequate to maintain body weight and did not compromise urea biosynthetic ability compared with dogfish fed a normal diet of chopped fish (Hazon and Henderson, 1984). Osmoregulatory ability was not, therefore, altered by the HPD and fish on this diet were considered to be the

control dietary group in this study. The LPD was not apparently an adequate dietary source of protein and appeared to impair the hepatic urea production rate of test animals by restricting the essential substrate for urea synthesis.

In normal seawater the urea BPR in LPD was approximately half that of the HPD fish. Plasma urea concentration (and thus plasma osmolality) was maintained by a significant reduction in urea MCR, to approximately half that of the HPD group. The urea MCR's observed in the LPD fish group are among the lowest ever measured in an elasmobranch and probably represent the unavoidable or obligatory urea loss. Thus, the LPD fish appear to have minimised urea loss across membranes involved in transport to the environment e.g. the kidneys and gills, presumably by decreasing membrane permeability.

In many non-mammalian vertebrates changes in membrane permeability are mediated by AVT. In birds, reptiles and amphibians AVT stimulates antidiuresis by decreasing GFR and increasing renal tubular water reabsorption and in eels antidiuresis is mediated by changes in GFR (Bentley, 1982). Elasmobranchs alter GFR by changing the number of filtering nephrons (Shannon, 1940: Kempton, 1953: Henderson, Oliver, McKeever and Hazon, 1978) and possibly also by altering individual nephron filtration rates (Brown and Green, 1987). They are also unusual among fish in their ability to alter renal tubular water permeability and it has been suggested that an antidiuretic principle may be involved

(Henderson, Oliver, McKeever and Hazon, 1978). Thus, there are two potential actions of AVT at the elasmobranch kidney. The first may be to decrease GFR which would reduce the filtered urea load and therefore decrease urea excretion. The second may be to increase tubular water permeability. Accepting the urea impermeability of all but the most distal region of the elasmobranch nephron (Friedman and Hebert, 1990), this change in tubular water permeability of the more proximal region would increase the urea concentration of the fluid remaining in the renal tubule. Consequently, upon entering the urea-permeable distal region, the urea concentration gradient from tubular fluid to interstitium would be increased thus allowing greater tubular urea reabsorption. This, together with a decrease in GFR would considerably minimise urea loss and would contribute significantly to the observed whole body decrease in urea MCR. Other potential sites of action for AVT are the rectal gland and gills. The rectal gland functions intermittently and its secretion contains large concentrations of salt but little urea. It is difficult, therefore, to envisage how, under normal circumstances, the actions of AVT could produce a significant decrease in urea loss at the rectal gland.

AVT affects the permeability of teleost gills by altering the distribution of gill blood flow and possibly by a direct effect on gill epithelium (Bentley, 1982). Changes in elasmobranch gill blood flow may account for some of the observed decrease in urea clearance but it is

more probable that an AVT-induced decrease in gill urea clearance would be a direct effect on the gill epithelium, further decreasing its permeability to urea.

AVT-mediated control of membrane permeability could be a general homeostatic mechanism by which elasmobranchs modify their urea retention. It would be especially important in conditions where large decreases in urea clearance were necessary to maintain plasma urea concentrations. Plasma AVT concentrations and GFR's were not measured in this study and clearly the changes suggested would need to be confirmed experimentally.

Effects on osmoregulation in 50% seawater

Fish on a HPD adapted to 50% seawater displayed decreased plasma osmolality as a result of decreased plasma urea and electrolyte concentration. The decrease in plasma urea concentration was mediated by an increase in urea clearance and a decrease in urea production. Plasma 1α-OH-B concentrations were significantly elevated in those animals.

Hazon and Henderson (1984) demonstrated that the decrease in plasma urea concentration was in direct proportion to the decrease in environmental osmolality whereas decrease in plasma sodium concentration occurred only to about 70% seawater after which its plasma concentration was maintained at this new lower level. A key observation made by Hazon and Henderson (1984) was that the body weights of animals adapting from 100% to 50% seawater changed during the course of the adaptation: body

weight increased by 20% during adaptation from 100% to 80% seawater and then subsequently decreased as adaptation continued, returning to its control value in 50% seawater. The increases in body weight were accompanied by changes in haematocrit, which showed a decrease to 80% seawater then recovery in 50% seawater. The increase in body weight was undoubtedly caused by osmotic water influx across the gills, which would produce hypervolaemia and possibly hypertension. This increase in body fluid volume appears to be tolerated to about 80% seawater whereafter homeostatic mechanisms acted to compensate.

In mammals and many other vertebrates, hypervolaemia is a classical stimulus for ANP release (Genest and Cantin, 1988; Inagami, 1989; Brenner, Ballerman, Gunning and Zeidel, 1990; Evans, 1990). ANP-like immunoreactivity has been demonstrated in atrial tissue from several species of elasmobranch (Evans et al, 1989; Uemura et al, 1990) and the amounts found were compatible to those observed in many teleost fish (Uemura et al, 1990). Furthermore, volume expansion in vivo produced significant stimulation of rectal gland secretion (Solomon et al, 1984, 1985a).

Based on the results obtained in this study it is proposed that the adaptation of dogfish on a HPD to 50% seawater is mediated in part by ANP. During the initial phase of adaptation hypervolaemia resulting from osmotic water influx is tolerated and body weight increases. In about 80% seawater the hypervolaemia can no longer be tolerated and ANP is released in response to atrial

distension (Solomon et al, 1984, 1985b). ANP would have several effects. Firstly, via a direct effect on vascular smooth muscle, ANP would cause vasodilation and a decrease in mean arterial blood pressure. This effect has been demonstrated in elasmobranchs both in vivo, using atriopeptin II (Solomon et al, 1985b) and human ANP (Hazon et al, 1987), and in vitro, utilising atriopeptin II (Solomon, Solomon, Silva and Epstein, 1985) and mammalian ANP (Evans and Weingarten, 1989). Secondly, by acting on the intrarenal vasculature ANP would cause changes in glomerular perfusion which may increase GFR producing a diuresis and natriuresis. This would decrease blood volume and contribute to the lowering of mean arterial blood pressure. Thirdly, ANP would stimulate rectal gland secretion by decreasing vascular resistance within the gland, thereby increasing gland blood flow (Solomon et al, 1985b) and possibly also by stimulating VIP release from neural stores within the gland (Silva et al, 1987). This increase in salt secretion, together with the natriuresis would produce a decrease in plasma electrolyte concentration. The diuresis would also increase renal urea excretion. Thus, in addition to causing a reduction in blood pressure and blood volume, ANP would also cause a decrease in plasma osmolality, by decreasing plasma sodium, chloride and urea concentrations, and thereby reduce the driving force for osmotic water influx. The combined effects of ANP would reduce the stimulus for its own

release and thus act as a classical negative feedback system.

ANP also stimulates 1α-OH-B secretion (see Figure 4.24) and may account for the increased plasma 1α-OH-B concentration observed in HPD fish in dilute media. Plasma 1α-OH-B concentration first shows an increase in approximately 70% seawater (Hazon and Henderson, 1984) which coincides with the point at which ANP would be predicted to have its maximal biological effect. Thus, it is possible that ANP acts as the initial stimulus for increased 1α-OH-B secretion. However, 1α-OH-B secretion remains elevated in 50% seawater, at which point there would no longer be a hypervolaemic stimulus for ANP secretion and consequently no elevated plasma ANP concentration. There must, therefore, be a sustained stimulus that mediates the long term elevation of plasma 1α-OH-B concentration in 50% seawater. 1α-OH-B is believed to function in the maintenance of plasma sodium concentration (Hazon and Henderson, 1984) and it may be sodium concentration which controls the sustained stimulus for elevated 1α-OH-B secretion. The nature of this stimulus is, however, unknown but one could postulate the existence of a central "salt receptor" which would respond to extreme changes in plasma sodium concentration by activating the hypothalamo-pituitary-interrenal axis, stimulating the secretion of 1α-OH-B via the release of ACTH. This mechanism could include a neuromodulatory influence by central peptides. It is interesting that ANP-

like immunoreactivity has been identified in the hypothalamus of Scyliorhinus canicula (Vallarino et al, 1990) and may operate in a manner similar to BNP (structurally similar to ANP) which was recently identified in mammals (Sudoh, Kangaura, Minamino and Matsyo, 1988) and teleosts (Takei, personal communication). A possible sequence of events could be as follows. Hypervolaemia would stimulate the release of ANP from atrial cardiocytes and the central release of a structurally similar BNP. ANP would have the peripheral effects described previously and would provide the initial stimulus for 1α-OH-B secretion. BNP would act centrally, possibly at the hypothalamus, and have a neuromodulatory role. This would involve potentiating or mediating the response to the putative salt receptor.

1α-OH-B may have several sites of action and receptor glycoproteins have been located in the gills, rectal gland, kidney and liver of elasmobranchs (Kane and Idler, 1980) and their presence confirmed in these tissues by immunofluorescence (Burton and Idler, 1986). The initial studies of Hazon and Henderson (1984), where plasma 1α-OH-B concentration was elevated, showed that in response to decreased osmolality, below 75% seawater, urea continued to be lost but plasma sodium concentration was regulated at a new lower level. Similar plasma sodium concentrations were measured in the current studies. The effects of 1α-OH-B could, therefore, be to stimulate a change in sodium transport at various epithelia. At tissues such as the

rectal gland and gills, which actively excrete sodium, the effect could be to decrease excretion, and at tissues such as the kidneys, which actively reabsorb sodium, the effect could be to increase reabsorption. These combined effects of 1α-OH-B could regulate and maintain plasma sodium concentration around a new lower level.

Thirdly, 1α-OH-B could act at the liver; Hazon and Henderson (1984) suggested that 1α-OH-B may depress plasma urea concentration by decreasing hepatic urea biosynthesis. This is not, however, supported by the results of this study. In both dietary groups in 50% seawater plasma 1α-OH-B concentrations were elevated and urea BPR's were decreased. However, there was no change in urea BPR in LPD fish adapted to 130% seawater, compared to 100% seawater control fish, and in these fish plasma 1α-OH-B concentration was also greatly increased. It is therefore suggested that the hepatic effect of 1α-OH-B is something other than an effect on urea synthesis.

Clearly some aspects of this hypothesis on the adaptation of dogfish on a HPD to a low osmotic environment would need to be confirmed experimentally: specifically, plasma ANP and ACTH concentrations would need to be monitored throughout the adaptation period and also the effects of ANP on the elasmobranch kidney would need to be assessed. The proposed effects of 1α-OH-B would also need to examined in the various target tissues. In particular a reappraisal of its effects on the rectal gland is required since there is conflicting evidence on the effects of

corticosteroids (Chan, Phillips and Chester-Jones, 1967; Holt and Idler, 1975). LPD fish adapted to 50% seawater show similar plasma composition changes to HPD fish. However, the increase in 1α-OH-B concentration is not as large as that observed in the HPD fish and the urea secretory dynamics are different. As a result of dietary protein restriction LPD fish have lower clearance rates in 100% seawater and this is possibly mediated via AVT. Because of the general decrease in membrane permeability possibly mediated by AVT, the hypervolaemia and hypertension experienced on adaptation to 50% seawater would be less severe than that of the HPD fish. Thus, the stimulation of ANP release would be less great, which may explain why the observed increase in plasma 1α-OH-B concentration was less than that observed in HPD fish.

Effects on Osmoregulation in 130% seawater

High and low protein diet fish showed markedly different plasma compositions in 130% seawater. HPD increased plasma osmolality by increasing plasma electrolyte and urea concentrations, the latter being achieved by a decrease in urea MCR. Plasma 1α-OH-B remained unchanged. LPD, however, adapted quite differently. Plasma urea concentration and urea dynamics remained unchanged from normal seawater values and plasma osmolality was increased by significantly increasing plasma sodium and chloride concentrations. In addition, plasma 1α-OH-B concentrations were also greatly increased to some of the highest levels ever reported in an elasmobranch.

Fish on the LPD were apparently not able to (1) synthesize more urea, presumably because of dietary protein restriction and (2) further decrease urea MCR, presumably indicating that the MCR in 100% seawater was minimal and no further decrease in permeability was possible. Furthermore, Hazon and Henderson (1984) demonstrated that the body weights of fish adapted to hyperosmotic environments gradually fell without any recovery, and that the haematocrits of these animals gradually increased, indicating that they were becoming hypovolaemic. Thus, it is probable that LPD fish in a high osmotic environment, which do not have the capacity to increase plasma urea concentration would experience severe hypovolaemia and possible hypotension. In mammals and other vertebrates hypotension is a classical stimulus for activation of the renin-angiotensin system and the release of AII. It has also been demonstrated that Scyliorhinus canicula responded to papaverine-induced hypotension by activation of an endogenous renin-angiotensin-like system which acted to correct the fall in blood pressure. It is possible, therefore, that in LPD fish in a high osmotic environment, experiencing severe hypovolaemia and hypotension, the endogenous renin-angiotensin system would be activated. AII could have several effects. Firstly, by either a direct effect on vascular smooth muscle or in a catecholamine release AII could stimulate an increase in blood pressure (Opdyke and Holcombe, 1976; Hazon, Balment, Perrott and O'Toole, 1989) which would ensure adequate

tissue perfusion. Secondly, AII could act to stimulate drinking which would (1) restore plasma volume and (2) increase plasma osmolality because of the high salt content of the ingested fluid. There is strong evidence for the dipsogenic effects of AII in elasmobranchs (Hazon, Balment, Perrott and O'Toole, 1989) and in the current study dissection of the LPD fish revealed large volumes of water in the gut, indicating that they had been drinking. Normally, a salt load in the absence of an increase in urea concentration would stimulate rectal gland salt secretion (Burger, 1962, 1965). This would be entirely inappropriate for adaptation to a high osmotic environment. Consequently, the third effect of AII could be to stimulate the secretion of 1α-OH-B which could inhibit rectal gland secretion. AII has been shown to be a potent steroidogenic factor both in vivo (Hazon and Henderson, 1985) and in vitro (Figures 4.20, 4.21) and would account for the very large observed increase in plasma 1α-OH-B concentration. The overall effects of 1α-OH-B would be to maintain and regulate plasma sodium concentration in similar manner to its effects in 50% seawater. The only difference in this case would be that plasma sodium concentration would be regulated around a new upper rather than lower level. Lacking the ability to increase plasma urea concentration LPD fish would constantly lose body weight (Hazon and Henderson, 1984) and experience hypovolaemia and hypotension. This would provide a sustained stimulus for AII production which in turn would act to counteract the

hypotension and hypovolaemia and provide the sustained stimulus for 1α-OH-B secretion. LPD fish in 130% seawater obviously represent an extreme case where activation of the endogenous renin-angiotensin system is essential for survival. However, the RAS might be the general mechanism by which euryhaline elasmobranchs could adapt from brackish to normal seawater. These animals would be unable to tolerate the acute hypovolaemia and hypotension unless they had a rapid mechanism for increasing blood pressure, plasma volume and osmolality (thus decreasing osmotic water loss). The activation of the endogenous RAS would ensure survival long enough for urea biosynthesis in fed fish to increase and elevate plasma urea concentration to a level that would maintain an iso- or hyper-osmotic plasma.

Certain aspects of this hypothesis would need to be confirmed experimentally. In particular the activation of components of the RAS, such as blood pressure and drinking effects, could be investigated using specific inhibitors, such as captopril. It would also be useful to measure plasma concentration of endogenous AII but to date no heterologous antiserum has been found to measure AII in elasmobranchs (Hazon, Balment, Perrott and O'Toole, 1989).

HPD fish in 130% seawater appear to have adapted to increased plasma osmolality by decreasing body clearance of urea and electrolytes. These changes, presumably resulting from altered membrane permeabilities, could be mediated by AVT which would act in a manner similar to that described for LPD fish in 100% seawater. AVT could stimulate

antidiuresis and decrease gill permeability, effects which would minimise water loss and increase plasma osmolality. Thus, the osmoregulation of HPD fish in 130% seawater may resemble that of LPD fish in 100% seawater. It is possible that activation of RAS may also occur in HPD fish but would be less marked than in LPD fish, because of their ability to increase plasma urea concentration and thus plasma osmolality.

In summary, fish on high and low protein diets showed differences in the way in which they osmoregulated in normal, 50% and 130% seawater. In normal seawater LPD fish were unable to maintain the same level of urea synthesis as HPD fish and plasma osmolality was maintained by a significant decrease in metabolic clearance, possibly mediated by AVT. In 50% seawater HPD fish decreased plasma osmolality by decreasing plasma urea and sodium concentrations. The changes in plasma composition were possibly mediated by ANP, which may also have stimulated 1α-OH-B secretion. The elevated plasma 1α-OH-B concentration may be involved in regulating plasma sodium concentration at the new lower level. The elevated plasma 1α-OH-B levels in LPD fish in 50% seawater were less than those in HPD fish and this may reflect the apparent lower membrane permeability of the LPD animals. In 130% seawater LPD fish apparently had no option but to drink seawater in order to maintain plasma volume and osmolality, and this was possibly controlled by AII. Unlike the HPD fish in 130% seawater which were able to increase plasma urea

concentration, LPD fish had extremely high plasma 1α-OH-B concentrations, the 1α-OH-B possibly functioning to regulate plasma sodium at the new upper level.

The dietary and osmotic adaptation experiments have proved useful in elucidating the possible roles of 1α-OH-B in elasmobranchs, but they have also generated questions as to the nature of the factors controlling elasmobranch corticosteroidogenesis. To date, no specific RIA's exist for elasmobranch ANP, because the peptide has not yet been sequenced, although a project is currently under way to do so (Takei and Hazon, personal communication). At the time of dietary adaptation experiments an assay for AVT was not available and consequently plasma AVT concentrations could not be measured. When an AVT assay subsequently became available there was insufficient blood plasma remaining from dietary adapted animals to use in the assay. However, it was possible to assess the effects of heterologous peptides on corticosteroidogenesis in vitro using an isolated perifused interrenal gland.

Isolated in vitro perifused adrenocortical preparations have been widely applied to study the mechanisms of actions of factors controlling secretion of corticosteroids. They give insight into second messenger systems involved in adrenocorticosteroid biosynthesis and have been applied to mammalian (Forster and Rasmussen, 1983; Kojima, Lippes, Kojima and Rasmussen, 1983; Kojima, Kojima, Kreutter and Rasmussen, 1984; Kojima, Kojima and Rasmussen, 1985a, b, c, d), amphibian (Leboulenger et al,

1978; Perroteau et al, 1985; Lihrmann et al, 1985, 1986, 1987; Benyamina et al, 1987) and teleostean (Gupta, Lahlou, Botella and Porthe-Nibelle, 1985; Decourt and Lahlou, 1986) corticosteroidogenesis. A recurring difficulty with this technique is tissue cellular homogeneity, since zonal problems arise in mammals and there is intermingling of "cortical" and "medullary" homologues in non-mammalian vertebrates (Chester-Jones and Mosley, 1980). Among the vertebrates, the elasmobranchs are especially useful in that the adrenal homologue (interrenal gland) is not split into zones or mixed with non-cortical cells (Chester-Jones and Mosley, 1980). The dogfish interrenal gland has proved a suitable preparation to study the control of corticosteroid production because of the homogeneity of the cortical cells and its relatively stable, spontaneous secretion of 1α-OH-B.

In this study the interrenal preparation was shown to remain viable and responsive to stimulation for at least 21 hours. In practice most of the experiments carried out lasted approximately half this time period and the glands were fully responsive to at least four doses of agonist.

Little is known of the control of elasmobranch interrenal function, although components of a pituitary-interrenal axis have been identified (Klesch and Sage, 1975; Denning-Kendal, Sumpter and Lowry, 1982). The present study has examined several potential mediators of steroidogenesis in elasmobranchs and some of the second messenger systems that may be involved.

ACTH has been shown to stimulate steroidogenesis in elasmobranchs and its effect may involve the synergistic action of both cAMP and Ca^{2+}. The evidence for cAMP involvement is as follows. Firstly, in the presence of cholera toxin (CTx) the steroidogenic effect of ACTH was potentiated. CTx is a highly specific agent which acts on the stimulatory G proteins (G_s) which activate adenylate cyclase. By "locking" G_s in the active GTP-bound state, CTx causes the persistent stimulation of adenylate cyclase. Secondly, forskolin, an activator of adenylate cyclase (Seamon, Padgett and Daly, 1981), also stimulated steroidogenesis. Both the above results point to the involvement of cAMP in the stimulation of steroidogenesis in elasmobranchs. This was confirmed by the action of the membrane soluble analogue of cAMP, dibutyryl cAMP, which produced a dose-dependent increase in 1α-OH-B secretion. The requisite parts of the cAMP messenger system have been identified in an elasmobranch and their role in steroidogenesis demonstrated. Clearly, definite evidence that ACTH operates via the cAMP messenger system could be obtained by measuring intracellular cAMP concentrations in isolated interrenal cells before and after ACTH stimulation and this is one obvious future experiment. From the results obtained in this study it is suggested that the corticosteroidogenic effect of ACTH in elasmobranchs is mediated by the cAMP messenger system as it is in mammals (Kojima, Kojima and Rasmussen, 1985b) and amphibians (Lihrmann et al, 1986). Calcium is also involved in the

steroidogenic effect of ACTH in elasmobranchs. Similar to the situation in mammals (Kojima, Kojima and Rasmussen, 1985b, c), ACTH-induced steroidogenesis in elasmobranchs is greatly decreased but not abolished in the absence of extracellular calcium. This differs from the situation reported in teleosts (Decourt and Lahlou, 1986) and amphibians (Lihrmann et al, 1986) where removal of extracellular calcium completely abolishes the effect of ACTH. Using the calcium ionophore, A23187, it has been demonstrated in this study that a calcium influx stimulates corticosteroid production. However, the mechanism by which calcium exerts its effects is unclear. In mammals calcium is believed to facilitate the binding of ACTH to its receptor (Cheitlin, Buckley and Ramachandran, 1985) and modulate the receptor-mediated activation of adenylate cyclase (Fakunding, Chow and Catt, 1979). In addition, calcium influx occurs during ACTH stimulation of corticosteroidogenesis in mammals (Kojima, Kojima and Rasmussen, 1985b, c) and amphibians (Lihrmann et al, 1986).

The resultant increase in intracellular calcium concentration has been linked with activation of calmodulin-dependent enzymes, which are believed to initiate corticosteroidogenesis (Kojima, Kojima and Rasmussen, 1985b), and with increased sensitivity of the adenylate cyclase system (Fakunding, Chow and Catt, 1979; Kojima, Kojima and Rasmussen, 1985b). In mammals ACTH-induced calcium influx is blocked by the calcium channel antagonist, nitrendipine (Kojima, Kojima and Rasmussen,

1985b) and in amphibians it is inhibited in a dose dependent manner by the voltage-dependent calcium channel antagonist, verapamil (Lihrmann et al, 1986).

However, the specificity of action of verapamil has been questioned since it also significantly inhibits the conversion of corticosterone to aldosterone in mammalian (Schiebinger, Braley, Menachery and Williams, 1983) and amphibian (Lihrmann et al, 1986) adrenocortical cells. In the present study on Scyliorhinus canicula, the use of a dose of verapamil, which produced significant inhibition in amphibians, had no effect on ACTH-induced steroidogenesis. This result may indicate that ACTH-induced calcium influx occurs through non-voltage-dependent calcium channels in elasmobranchs and/or it may simply reflect the fact that the enzymes responsible for converting corticosterone to aldosterone are not present in elasmobranchs. One final possibility is that ACTH-induced steroidogenesis in elasmobranchs does not involve the influx of calcium, although this does seem unlikely given the important role of calcium in other groups. Perhaps the best way in which to assess Ca^{2+} influx during ACTH-stimulation of steroidogenesis could be to measure the influx directly using radiolabelled $^{45}CaCl_2$ (Kojima, Kojima and Rasmussen, 1985b). This would allow the rate of influx to be quantified and permit a clearer assessment of the effects of calcium antagonists, such as verapamil.

In summary, it appears that the mechanism of ACTH-induced steroidogenesis in elasmobranchs may be similar to

that reported for mammals and amphibians. Stimulation of secretion appears to involve the cAMP messenger system and is largely dependent on the presence of extracellular calcium but ACTH does not induce the release of calcium from intracellular calcium pools.

AII stimulates corticosteroid secretion in Scyliorhinus canicula. Of the two AII's used in this study Ile^5-AII was considerably more potent than Val^5-AII. An endogenous elasmobranch AII-like molecule has not yet been identified but the significant difference in potencies of Val^5 and Ile^5-AII could suggest that Ile^5-AII may have greater homology with the putative endogenous AII-like molecule.

In mammals and amphibians the steroidogenic effect of AII is believed to be mediated by the inositol phosphate messenger system and requires both extracellular and intracellular calcium (Kojima, Kojima, Kreutter and Rasmussen, 1984; Kojima, Kojima and Rasmussen, 1985a, c, d; Lihrmann et al, 1986, 1987). The sequence of events that are believed to follow AII binding to its receptor are complex and have been described fully in section 1.6.2e and Figure 1.8b.

In the present study it has been demonstrated that AII-induced steroidogenesis in elasmobranchs requires both intracellular and extracellular calcium and may operate in a manner similar to that reported in mammals and amphibians. In the presence of dantrolene, AII-induced steroidogenesis in interrenal cells was significantly inhibited in Scyliorhinus canicula (Figure 4.23), a result

that has also been reported in mammalian (Kojima, Kojima, Kreutter and Rasmussen, 1984; Kojima, Kojima and Rasmussen, 1985d) and amphibian (Lihrmann et al, 1987) adrenocortical cell preparations. Dantrolene inhibits steroidogenesis by blocking the release of calcium from intracellular calcium pools. The release of calcium from intracellular stores is responsible for initiating the secretory response to AII in mammals and amphibians and is believed to be mediated by the direct action of IP_3 (Kojima, Kojima and Rasmussen, 1985d). The results of the present study suggest that the same may also be true in an elasmobranch. To confirm the involvement of the inositol phosphate second messenger system it would be necessary to demonstrate an increase in the intracellular IP_3 concentration of interrenal cells in response to stimulation by AII.

In the absence of extracellular calcium, AII-induced corticosteroidogenesis was also significantly inhibited in Scyliorhinus canicula (Figure 2.22). A similar effect has also been observed in both mammalian (Kojima, Kojima and Rasmussen, 1985a) and amphibian (Lihrmann et al, 1987) adrenocortical preparations. Extracellular calcium is believed to be required for the sustained phase of corticosteroid secretion, during which the rates of calcium influx and calcium efflux are both increased, i.e. the rate of calcium cycling across the cell membrane is increased (Kojima, Kojima, and Rasmussen, 1985d). This rate is important because it controls the turnover rate of the DG-activated, calcium-dependent protein kinase C which

controls the sustained phase of AII-induced steroidogenesis. The results of the present study suggest that AII may also increase the rate of calcium cycling across the cell membrane of elasmobranch interrenal cells. This study has also shown that the calcium influx component of calcium cycling may not be mediated through voltage-dependent calcium channels since verapamil had no apparent effect on the response to AII stimulation.

Taken together, the effects of dantrolene and calcium-free media on AII-induced corticosteroidogenesis in elasmobranchs suggest that the mechanism of action of AII is similar to that reported for mammals (Kojima, Kojima and Rasmussen, 1985d) and amphibians (Lihrmann et al, 1987). Clearly, however, further experiments are required to clarify certain aspects of AII-induced steroidogenesis in elasmobranchs. Measurements of intracellular calcium concentration during stimulation would be useful as it would confirm the initial transient increase in calcium concentration (attributable to IP_3 stimulation) and the constant, non-elevated calcium concentration during the sustained phase of AII stimulation. Calcium efflux and influx could also be measured during the course of AII stimulation using radioactive ^{45}Ca tracer (Kojima, Kojima and Rasmussen, 1985a, d). Radiolabelled precursors of IP_3 and DG biosynthesis could also be used to monitor the concentrations of these messengers within elasmobranch interrenal cells during AII stimulation.

Although there is good evidence for the existence of ANP in elasmobranchs, the actual peptide sequence is not known and is currently been investigated (Takei, personal communication). In the current studies it was necessary to use synthetic $ANP_{(101-126)}$. $ANP_{(101-126)}$ significantly stimulated 1α-OH-B secretion by the dogfish interrenal gland. This is in contrast to its effects in mammals where it either decreased (Atarishi et al, 1984; De Lean et al, 1984; Goodfriend et al, 1984; Kudo and Baird, 1984) or had no effect (Chartier, Schiffrin, Thibault and Garcia, 1984; Campbell, Currie and Needleman, 1985) on basal corticosteroid production. Similarly, in the amphibian, Rana ridibunda, $ANP_{(101-126)}$ had no effect on basal corticosteroid synthesis (Lihrmann et al, 1985). The above studies also showed that $ANP_{(101-126)}$ reduced the response of adrenal glomerulosa cells to corticotropic factors, such as ACTH and AII. The effect of $ANP_{(101-126)}$ on the response of the interrenal gland to other steroidogenic agents was not tested in the present study and is obviously an experiment for the future.

The steroidogenic effect of ANP on the elasmobranch interrenal is consistent with its proposed role in the adaptation of elasmobranchs to dilute osmotic environments. ANP is known to operate via the cGMP messenger system and ANP inhibition of aldosterone secretion is associated with an increase in intracellular cGMP concentration in rat adrenal cells (Matsuoka et al, 1985). In the present study it has been demonstrated that the membrane permeable

analogue of cGMP, dibutyryl cGMP, stimulates 1α-OH-B secretion from elasmobranch interrenal cells in a dose-dependent manner. It is probable, therefore, that the mechanism of the novel ANP-induced stimulation of corticosteroidogenesis in elasmobranchs is mediated via the cGMP messenger system. Clearly direct proof of this could only be obtained by demonstrating an increase in the cGMP concentration of elasmobranch interrenal cells during ANP stimulation. AVT stimulated steroidogenesis in the elasmobranch interrenal gland and is reported to have a similar effect on the amphibian adrenal (Larcher et al, 1989). However, AVT was the least potent of the peptide hormones tested in this study and the concentration of AVT used to stimulate the interrenal gland in vitro was at least several orders of magnitude higher than the reported plasma AVT levels measured in other vertebrates (Bakker and Bradshaw, 1978; Stallone and Braun, 1985). It is unlikely that the circulating concentrations of AVT in elasmobranch plasma would be sufficient to significantly stimulate corticosteroidogenesis in vivo. It should also be stressed that this experiment examined the effect of AVT in isolation, a situation never encountered by the interrenal gland in vivo, and it is likely that in vivo the effects of AVT on the interrenal gland would be modified by other circulating hormones.

In the current studies an increased potassium concentration in the perifusion medium caused an increase in steroid production which was significant only at 28mM, a

concentration well beyond the physiological range of an elasmobranch. In mammals, potassium is well known to control aldosterone and corticosterone production by zona glomerulosa tissue, but not cortisol or corticosterone production by inner zone tissue (Haning, Tait and Tait, 1970). In amphibians, however, steroid production could be stimulated only by very high concentrations of potassium (Maser, Janssens and Hanke, 1982) and no effect was observed at potassium levels capable of increasing mammalian production rates (Lihrmann et al, 1985). In the isolated perifused interrenal gland (head kidney) of the trout, Salmo gairdneri, increased potassium concentrations had no effect on cortisol output.

In the teleost fish, Salmo gairdneri, cortisol production was altered in response to changes in extracellular sodium concentration (Decourt and Lahlou, 1987). Conversely, in the present study the elasmobranch interrenal gland appeared unresponsive to increased sodium concentration in the perifusion medium and a previous study by O'Toole (1987) demonstrated that the interrenal gland was also unresponsive to decreased sodium concentration in the perifusion medium. These are interesting results because they indicate that the increases in plasma 1α-OH-B concentrations measured in the dietary osmotic adaptation experiments are not the result of a direct sodium effect on the interrenal gland itself, and support the idea that other (probably hormonal) mediators are involved in the control of 1α-OH-B secretion during osmotic adaptation.

The isolated in vitro perifused adrenal/interrenal gland is an established technique which has been widely used to study the mechanisms of vertebrate corticosteroidogenesis. The current studies have demonstrated that this technique is a valuable tool with which to study elasmobranch corticosteroidogenesis. One advantage of the elasmobranch interrenal preparation over other vertebrate preparations is the homologous nature of the interrenal tissue. This simplifies, to some extent, the interpretation of results since no account need be taken of interfering factors released from contaminating cell types (such as chromaffin and renal tissue), which is a common problem in amphibian preparations (Delarue et al., 1988; Leboulenger et al., 1988). The current studies have provided the first insight into the factors which may control corticosteroidogenesis in elasmobranchs and to some extent to some of the second messengers that may be involved in mediating the responses. There is, however, considerable scope for future experimentation using this technique and further studies could include:

(1) Investigation of other potential secretagogues. This would include (a) the prostaglandins E_2, I_2 and F_2alpha which are steroidogenic in amphibian interrenals (Delarue et al., 1986); (b) the other selachian neurohypophysial peptides, aspartocin and valitocin; (c) the uropophysial peptides. Immunohistochemical data provides firm evidence for the existence of peptides structurally related to teleost urotensin I and urotensin

II in the elasmobranch caudal neurosecretory system, but these peptides have not yet been purified from an elasmobranch. There is, however, an ongoing project to extract, sequence and synthesise elasmobranch urotensins (Conlon, personal communication); (d) catecholamines. The possible relationship between chromaffin and interrenal tissue has not been investigated in elasmobranchs. This would be interesting since catecholamines, which stimulate corticosteroidogenesis in other vertebrates, are present in comparatively high concentrations in elasmobranch plasma (Butler, Capra and Davidson, 1978; Butler, Taylor and Davidson, 1979).

(2) Investigation of the interactions between potential secretagogues. This would attempt to simulate the situation <u>in vivo</u>, where the overall interrenal response results from a combination of circulating hormones which may be agonistic or antagonistic. For example, in amphibians the corticosteroidogenic effects of VIP and 5-hydroxytryptamine are potentiated by AVT (Larcher, Delarue, Vandesande and Vaudry, 1990).

In conjuction with the <u>in vitro</u> perifusion it would be useful to develop an isolated interrenal preparation. This technique permits the measurement of individual second messenger concentrations, e.g. inositol phospholipids by radiolabelling and HPLC, and calcium by microfluorimetry. It would also enable membrane and intracellular ion fluxes to be studied, e.g. calcium fluxes could be measured using $^{45}CaCl_2$ as tracer. Together with the use of specific

agonists and antagonists this technique would permit the clarification of the messenger system (or systems) involved in mediating the stimulatory response of a particular agonist.

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Appendix 1. Amino Acid Abbreviations

One-letter	Three-letter	Name
A	Ala	Alanine
C	Cys	Cysteine
D	Asp	Aspartic acid
E	Glu	Glutamic acid
F	Phe	Phenylalanine
G	Gly	Glycine
H	His	Histidine
I	Ile	Isoleucine
K	Lys	Lysine
L	Leu	Leucine
M	Met	Methionine
N	Asn	Asparagine
P	Pro	Proline
Q	Gln	Glutamine
R	Arg	Arginine
S	Ser	Serine
T	Thr	Threonine
V	Val	Valine
W	Trp	Tryptophan
Y	Tyr	Tyrosine

Appendix 2. Composition of Dogfish Ringer Solution

	Concentration ($mmol l^{-1}$)
NaCl	240.0
KCl	7.0
$CaCl_2$	10.0
$MgCl_2$	4.9
$NaHCO_3$	2.3
$Na_2HPO_4 2H_2O$	0.5
Na_2SO_4	0.5
Urea	360.0
TMAO	60.0
Glucose	1.0%

Appendix 3. Publications arising from the current studies

Secretory patterns of 1α-hydroxycorticosterone in the isolated perifused interrenal gland of the dogfish, *Scyliorhinus canicula*

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ABSTRACT

An isolated in-vitro perifused interrenal gland preparation from the dogfish *Scyliorhinus canicula* was used to study production of quantitatively the major corticosteroid 1α-hydroxycorticosterone (1α-OH-B), measured by radioimmunoassay. Basal secretory rates were 877·1 ± 145 (S.E.M.) fmol/mg per 15 min ($n = 14$) and the preparation remained viable for up to 22 h, as reflected in a brisk response to 10 μM cyclic AMP (cAMP) after this time. Steroid production responded in a dose-dependent manner to porcine ACTH, with 10 μM producing a maximum stimulation of 225% above the basal secretory rate. cAMP (10 μM) produced an increase of 278% above basal, while 1 μM forskolin increased basal secretory rates by 127%. [Val^5]- and [Ile^5]-angiotensin II (0·1 μM) increased 1α-OH-B production 120 and 372% respectively over basal secretory rates. Increasing the concentration of K^+ in the perfusate from 8 mM to 12, 18, 28 and 40 mM produced a significant rise only at 28 mM. Alterations in the concentration of Na^+ and osmolarity of the perifusion medium had inconsistent effects on steroid production. Increased concentrations of urea (from 360 to 720 mM) increased the basal secretory rate by 121%, whilst reducing the concentration of urea (from 360 to 90 mM) had no effect.

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INTRODUCTION

Isolated in-vitro perifused adrenocortical preparations are widely applied to study the mechanisms of action of factors controlling secretion of corticosteroids. They give insight into second messenger systems involved in adrenocorticosteroid biosynthesis and have been applied to mammalian (Kojima, Kojima & Rasmussen, 1985*a*, *b*, *c*), amphibian (Lihrmann, Netcitailo, Leboulenger *et al.* 1985; Benyamina, Leboulenger, Lihrmann *et al.* 1987) and teleostean (Decourt & Lahlou 1986) corticosteroidogenesis. A recurring difficulty with the technique is tissue cellular homogeneity, since zonal problems arise in mammals, and there is intermingling of 'cortical' and 'medullary' homologues in non-mammalian vertebrates (Chester Jones & Mosley, 1980). Among the vertebrates, the elasmobranch fish are especially useful in that the adrenal homolog (interrenal gland) is not split into zones or mixed w non-corticoid cells (Chester Jones & Mosley, 198 and its principal corticosteroid is the novel hormo 1α-hydroxycorticosterone (1α-OH-B) (Idler & Tru cott, 1966; Kime, 1977; Hazon & Henderson, 198

Little is known of the control of elasmobran interrenal function, although components of pituitary-interrenal axis have been identifi (Klesch & Sage, 1975; Denning-Kendall, Sump & Lowry, 1982). Possible non-pituitary control corticosteroidogenesis is complicated by the ported lack of a renin-angiotensin system (RA (Nishimura, Oguri, Ogawa *et al.* 1970), althou there is evidence that these fish possess the requis enzymes (Opdyke & Holcombe, 1976; Henders Oliver, McKeever & Hazon, 1981).

Mineralocorticoid actions of 1α-OH-B have be

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reported in heterologous bioassays (Grimm, O'Halloran & Idler, 1969), but in-vivo interpretation of these results is complicated by the unusual mode of osmoregulation seen in elasmobranch fish, depending as it does upon the retention of urea in the extracellular fluids rendering them iso- or mildly hyperosmotic to their marine environment (Smith, 1936). A possible role for 1α-OH-B in the control of urea biosynthesis has been suggested (Hazon & Henderson, 1984).

The present study applies an isolated perifused interrenal gland preparation from the lesser spotted dogfish *Scyliorhinus canicula* to the study of secretory patterns of 1α-OH-B and the regulation of its biosynthesis.

MATERIALS AND METHODS

Animals

Adult female dogfish (*Scyliorhinus canicula*), weighing about 800 g, were caught off the coast of Bangor, Gwynedd, U.K. They were maintained in aerated seawater (osmolarity 975 mOsm/1, pH 6·2–7·0; 405 mM Na^+, 9 mM K^+, 14 mM Ca^{2+}, 50 mM Mg^{2+} and 383 mM Cl^-) at 8–12 °C and fed once a week on chopped fish. Fish were preinjected with porcine adrenocorticotrophic hormone (ACTH; 110 nmol/kg body weight; Ferring AB, Malmo, Sweden) 24 h before the experiment. After decapitation the interrenal gland was removed, weighed and kept in ice-cold elasmobranch Ringer's solution (240 mM NaCl, 7·0 mM KCl, 10·0 mM $CaCl_2$, 4·9 mM $MgCl_2$, 2·3 mM $NaHCO_3$, 0·5 mM $Na_2HPO_4.2H_2O$, 0·5 mM Na_2SO_4, 360 mM urea, 60·0 mM trimethylamine oxide, 0·2% lactic acid and 1·0% glucose; osmolarity 1000 mOsm/1, pH 7·2).

Perifusion protocol

Each gland was diced into approximately 1 mm fragments and placed between two layers of gauze in a modified 5ml polythene syringe. Perifusion chambers were kept in a water bath maintained at 14 °C. Oxygenated perifusion media were delivered through Portex polythene tubing (Portland Plastics, Kent, U.K.) from a reservoir also maintained at the same temperature. Flow rates were $0·5 \pm 0·05$ ml/min. Glands were allowed to equilibrate for 2 h before introduction of test substances, which were added following a control period of 40–60 min. Effluent perifusates were collected every 10 or 15 min. At the end of each experiment, glands were challenged with a standard dose of 10 μM cyclic AMP (cAMP) to test their viability.

Test substances

Secretagogues were dissolved in Ringer's immediately before use. Dibutyryl cAMP, f [Ile^5]-angiotensin II ([Ile^5]-AII) and [V were obtained from Sigma (Poole, Dorset When individual solutes (Na^+, K^+ and ur altered the perifusion medium was maintain osmolarity of 1000 mOsm/1 by addition of r where necessary.

Steroid radioimmunoassay

Concentrations of 1α-OH-B were measu radioimmunoassay of its derivative 1-dehyd costerone as previously described (Kime Hazon & Henderson, 1984, 1985). Th sensitivity was 0·5 fmol, and the interas intra-assay coefficients of variation were 13% respectively.

Analysis of results

Individual collections were assayed and expr the percentage change in the basal secret defined as the mean of four collection immediately before the introduction of substance. Responses were tested for sign using a Mann–Whitney U test.

RESULTS

Validation of the preparation

During the initial 2 h of perifusion there wa wash-out of steroid as illustrated by Fig. 1 tinued perifusion demonstrated that th remained viable for long periods and v responsive to a dose of 10 μM cAMP af (Fig. 1*b*). After the wash-out period a stea basal secretion rate of 877 ± 145 (S.E.M.) fmol 15min ($n=14$) was established during th hour. Increasing doses (10 nM–10 μM) of ACTH produced a dose-dependent increas basal secretory rate of 1α-OH-B (Fig. 2), m stimulation being 225 ± 52% above basal (P A standard dose of 10 μM cAMP proc significant ($P<0·01$; $n=14$) increase above t 1α-OH-B secretory rate (Fig. 3). Forskoli also produced a significant ($P<0·05$) rise a basal secretory rate (Fig. 3).

Effect of angiotensin on steroidogenesis

[Val^5]-AII and [Ile^5]-AII (0·1 μM) sign ($P<0·05$) increased the secretion rate of 1 above basal (Fig. 4).

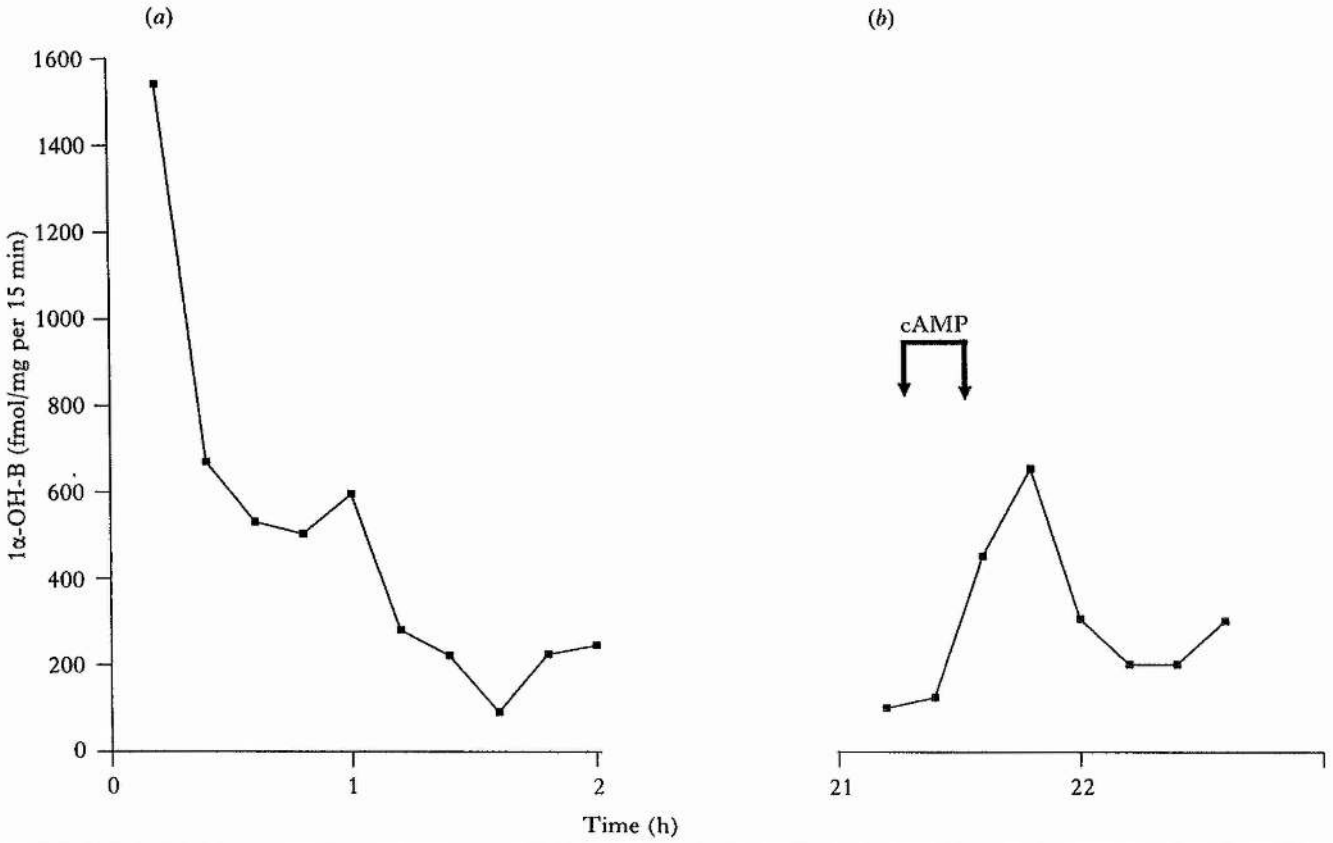

FIGURE 1. Mean secretory rates of 1α-hydroxycorticosterone (1α-OH-B) from two dogfish interrenal glands perifused over a 22-h period, showing (*a*) initial 'wash-out' of steroid for 2 h before attaining basal secretory rates, and (*b*) the effect of 10 μM cyclic AMP (cAMP) on 1α-OH-B production after 21 h.

Effect of changes in the composition of the perifusion medium

Increasing the concentration of K^+ in the perifusion medium (8 mM to 12, 18, 28 and 40 mM) produced a significant increase in secretory rate only at 28 mM (Fig. 5). Increasing the concentration of Na^+ (240 to 480 mM) did not alter the secretory rate of 1α-OH-B. An equivalent reduction in Na^+ (240 to 60 mM), rendered iso-osmotic by addition of mannitol, had no effect on the basal secretory rate. Similarly, increased or decreased osmolarity, whilst inducing perturbations in secretory rate, gave no consistent changes. Increasing the urea levels (360 to 720 mM), while maintaining osmolarity by reducing the Na^+ levels (240 to 60 mM), produced a consistent increase in 1α-OH-B levels to $120 \pm 34\%$ above the basal secretory rate ($P < 0{\cdot}001$; $n = 4$). Basal secretory rates remained constant during iso-osmotic reduction of urea levels (360 to 90 mM).

DISCUSSION

The dogfish interrenal gland has proved a suitable preparation for the study of the control of corticosteroid production; the gland consists of homogen ous cortical cells, is uncontaminated by chromaff or renal tissue and, in this preparation, produc stable spontaneous secretion of 1α-OH-B. The studies address mechanisms involved in the contr of secretion and biosynthesis of 1α-OH-B in t dogfish.

The present study suggests that when the elasm branch interrenal gland is challenged with heter logous ACTH, the intracellular mechanisms i volved in promoting steroidogenesis may be simil to those seen in other vertebrate systems and inclu adenylate cyclase and cAMP. Perhaps because of t heterologous nature of the ACTH, the sensitivity this preparation is somewhat less than that adrenocortical preparations from other vertebrat such as amphibia (Delarue, Tonon, Leboulenger *al.* 1981).

Previous studies on the dogfish interrenal gla have indicated that there is extra-pituitary control 1α-OH-B secretion *in vivo*, since hypophysector ized fish displayed reduced, but measurable, 1 OH-B in plasma for up to 12 months after operati (Hazon & Henderson, 1984). The RAS, if prese could possibly exert such control, and both mar

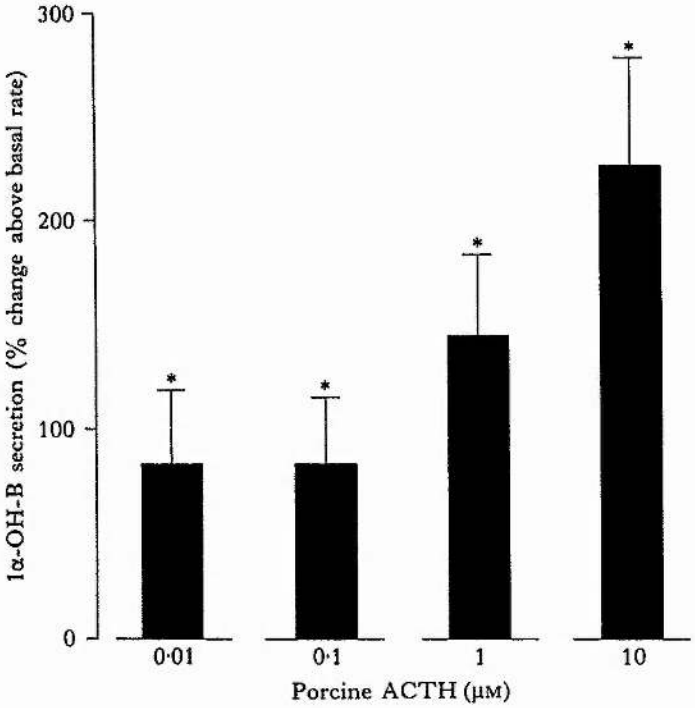

FIGURE 2. Effect of increasing doses of porcine ACTH on 1α-hydroxycorticosterone (1α-OH-B) production by perifused dogfish interrenal glands. Each gland was allowed 2 h to recover between each challenge. Results are means ± S.E.M. ($n=4$ or 5 as indicated below) of 1α-OH-B production expressed as percentage increases above the basal secretory rate. Mean basal secretory rates were: (i) for 0·01 μM ACTH, 6236 ± 436, 5157 ± 545, 6174 ± 545 and 6236 ± 352 fmol/mg per 15 min; (ii) for 0·1 μM ACTH, 4313 ± 506, 6404 ± 516, 3692 ± 144 and 5165 ± 1568 fmol/mg per 15 min; (iii) for 1 μM ACTH, 5701 ± 469, 4631 ± 684, 5168 ± 830, 5856 ± 644 and 449 ± 21 fmol/mg per 15 min; (iv) for 10 μM ACTH, 5490 ± 650, 4862 ± 875, 4862 ± 840 and 321 ± 73 fmol/mg per 15min. $*P<0·05$ compared with basal secretory rate (Mann-Whitney U test).

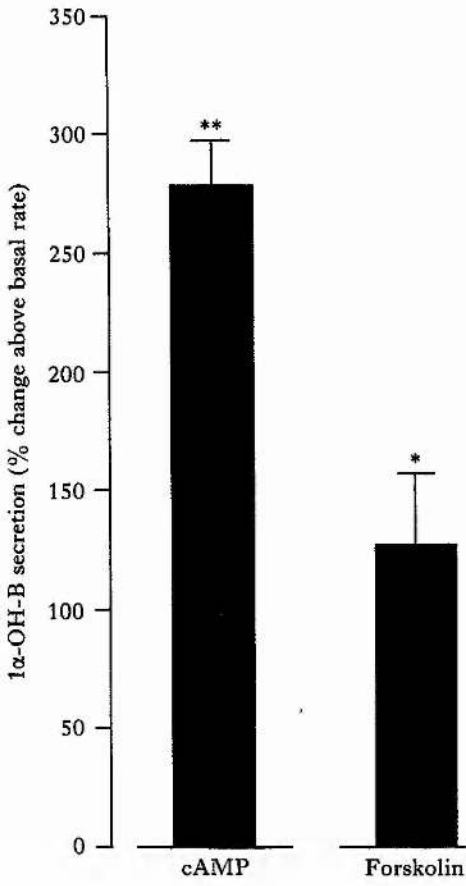

FIGURE 3. Effect of 10 μM dibutyryl cyclic AMI
$n=14$) and 1 μM forskolin ($n=3$) on 1α-hydrox
costerone (1α-OH-B) production by perifused
interrenal glands. Results are means ± S.E.M. of
production expressed as percentage increases a
basal secretory rate. Mean basal secretory rate
10 μM dibutyryl cAMP, 877 ± 145 fmol/mg per
(mean ± S.E.M., $n=14$); for 1 μM forskolin, 441
1364 ± 179 and 2210 ± 612 fmol/mg per 15 min
vidual glands. $*P<0·05$, $**P<0·01$ compared
secretory rate (Mann–Whitney U test).

malian AII and homologous renal extracts increase 1α-OH-B concentrations *in vivo* (Hazon & Henderson, 1985).

The stimulatory effect of AII on adrenal steroid release is relatively uniform throughout the vertebrates. In mammals, at least, the process seems to involve a calcium-dependent mechanism (Fakunding, Chow & Catt, 1979; Foster, Lobo, Rasmussen & Marusic, 1981). The increase in intracellular calcium both activates calmodulin-dependent enzymes to initiate the steroidogenic response and, in conjunction with diacylglycerol, controls the sustained production rate.

In the present studies, both AII analogues increased the secretory rate of 1α-OH-B. This is particularly interesting, since there is still disagreement as to the existence of a functional elasmo-

branch RAS (Nishimura *et al.* 1970; Hen
al. 1981). The responses to the peptide
used also generate some interesting questio
molecules so far sequenced, [Val^5]-AII
throughout the non-mammalian vertebra
& Peart, 1956; Nakayama, Nakajima &
1973, 1977), while [Ile^5]-AII, the mo
stimulator of 1α-OH-B secretion in th
preparation, is found in man and a f
mammals (Arakawa, Nakatani, Minohara
mura, 1967; Sokabe & Nakajima, 1972.

In mammals, potassium is well known
aldosterone and corticosterone productio
glomerulosa tissue, but not cortisol or corti
production by inner zone tissue (Hanin

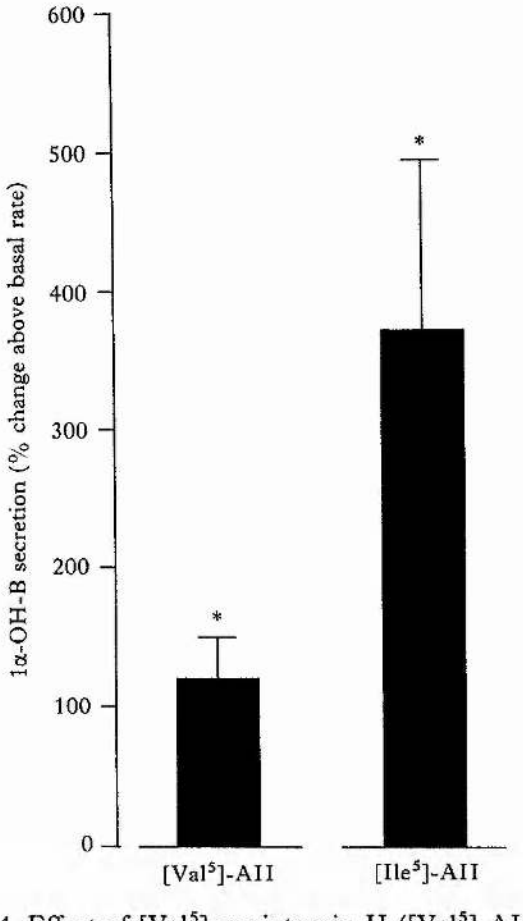

FIGURE 4. Effect of [Val^5]-angiotensin II ([Val^5]-AII) and [Ile^5]-AII (both 0·1 μM) on 1α-hydroxycorticosterone (1α-OH-B) production by perifused dogfish interrenal glands. Results are means±S.E.M. (n=4 or 5 as indicated below) of 1α-OH-B production expressed as percentage increases above basal secretory rates. Mean basal secretory rates for 0·1 μM [Val^5]-AII (n=5) were 2722±379, 2752±188, 5020±115, 4432±342 and 465±18 fmol/mg per 15min; mean basal secretory rates for 0·1 μM [Ile^5]-AII (n=4) were 311±61, 598±142, 1363±179 and 616±114 fmol/mg per 15 min. *P<0·05 compared with basal secretory rate (Mann–Whitney U test).

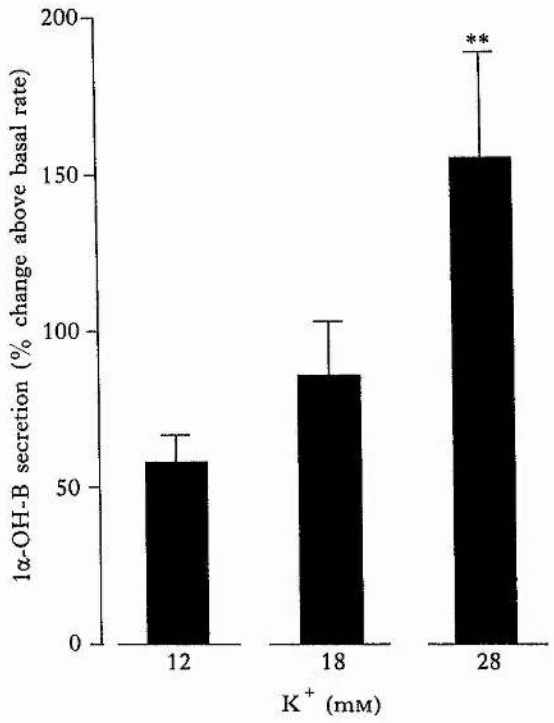

FIGURE 5. Effect of increasing doses of K^+ on 1α-hydroxycorticosterone (1α-OH-B) production by perifused dogfish interrenal glands. Results are means±S.E.M. (n=4) of 1α-OH-B production expressed as percentage increases above basal secretory rates. Mean basal secretory rates were: (i) for 12 mM K^+, 3564±238, 1926±205, 3699±628 and 4783±899 fmol/mg per 15 min; (ii) for 18 mM K^+, 3902±206 2420±413, 2848±374 and 7003±344 fmol/mg per 15 min; (iii) for 28 mM K^+, 3496±178, 4375±272, 4753±211 and 7732±762 fmol/mg per 15min. **P<0·01 compared with basal secretory rate (Mann–Whitney U test).

Tait, 1970). In amphibians, however, steroid production could be stimulated only by very high concentrations of potassium (Maser, Janssens & Hanke, 1982) and no effect was observed at potassium levels capable of increasing mammalian production rates (Lihrmann *et al.* 1985). In the isolated perifused interrenal gland (head kidney) of the trout, *Salmo gairdneri*, increased potassium concentrations had no effect on cortisol output (Decourt & Lahlou, 1986). In the current studies an increased K^+ concentration in the perifusion medium caused an increase in steroid production, but this was statistically significant only at the relatively high concentration of 28 mM, well beyond the normal physiological range.

A definitive role for 1α-OH-B in the control of th electrolyte economy of the dogfish is far from clea When studying animals with such an unusu osmoregulatory physiology as the elasmobranch care must be taken not to force accepted mineral corticoid roles upon its hormones. Thus in th present study, 1α-OH-B appears insensitive direct changes in extracellular osmolarity and Na concentrations. Conversely, in the teleost fis *S. gairdneri*, cortisol is responsive to both the factors (Decourt & Lahlou, 1986). In the prese study, however, increased levels of urea produced consistently large stimulation of the rate of secretio of 1α-OH-B *in vitro*. Previous studies (Hazon Henderson, 1984) on dogfish adapted to enviro ments of differing salinity showed that anima maintained their plasma osmolarities close to tho of their external medium and did so primarily at th expense of their plasma urea levels. The same stu showed that, in dilute environments, reduced pr

duction rates of urea were accompanied by increased plasma concentrations, and increased metabolic clearance rates of 1α-OH-B. The reverse was observed when fish were adapted to increased environmental salinities. 1α-OH-B may thus be concerned with the maintenance of plasma urea levels, possibly by acting on hepatic urea biosynthesis.

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63 CHANGES IN 1α-HYDROXYCORTICOSTERONE PRODUCTION IN RESPONSE TO REDUCED DIETARY PROTEIN AND OSMOTIC STRESS IN THE DOGFISH, *SCYLIORHINUS CANICULA*

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Elasmobranch fish possess a unique corticosteroid hormone 1α-hydroxycorticosterone (1α-OHB). The function of this hormone is not known although it may be related to the unusual method of osmoregulation, in which elasmobranchs maintain high levels of electrolytes and urea to maintain their plasma osmolality greater than the environmental sea water. 1α-OHB has been related to changes occurring in both urea metabolism and plasma sodium concentration during osmotic stress (Hazon & Henderson, 1984. J. Endocr. 103, 205-211). Furthermore, dietary protein restriction reduced hepatic urea production rate and also plasma 1α-OHB concentration (O'Toole & Hazon, 1989. J. Endocr. 121 suppl, 295). The current study exploits this model by investigating the role of 1α-OHB in fish with restricted protein intake adapted to a high (130% sea water) or a low (50% sea water) osmotic environment. Metabolic clearance rate (MCR) and blood production rate (BPR) of 1α-OHB were determined by constant isotopic infusion of ^{3}H-1α-OHB and plasma concentration by RIA. Both high and low protein diet fish adapted to 50% sea water showed significantly elevated plasma 1α-OHB levels (129.9±30.6 nmol/l vs. 55.5±12.7 nmol/l & 85.8±16.5 nmol/l vs. 37.8±5.5 nmol/l, respectively, $p<0.05$ in both cases). In both groups plasma Na^{+} was regulated at a lower level (186±5 nmol/l vs. 272±8 nmol/l & 181±5 vs. 271±5 nmol/l, respectively, $p<0.001$ in both cases) and plasma urea was significantly reduced (119±8 nmol/l vs. 285±6 nmol/l & 132±4 nmol/l vs. 295±3 nmol/l, respectively, $p<0.001$ in both cases). High protein diet fish adapted to 130% sea water showed no change in plasma 1α-OHB level. In contrast, low protein diet fish showed a significant increase (243.3±15.2 nmol/l, $p<0.01$) and this was achieved by a decrease in MCR (26.5±2.7 ml/h/kg vs. 71.6±10.1 ml/h/kg, $p<0.01$) and an increase in BPR (7.1±2.1 nmol/h/kg vs. 2.5±0.2 nmol/h/kg, $p<0.05$) of 1α-OHB. These fish also had greatly elevated plasma Na^{+} (343±9 nmol/l, $p<0.01$) but not plasma urea and this may indicate a role for 1α-OHB in Na^{+} and electrolyte balance.